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**AN EXAMINATION OF THE HYPOTHALAMO-
NEUROHYPOPHYSIAL SYSTEM OF THE RAT:
Restoration of the Vasopressinergic system**

A Dissertation Presented
by
LYNN M. DiBENEDETTO

Submitted to the Graduate School of Biomedical Sciences of
the University of Massachusetts Medical Center in partial fulfillment
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 1997

Department of Cell Biology

**AN EXAMINATION OF THE HYPOTHALAMO-
NEUROHYPOPHYSIAL SYSTEM OF THE RAT:**

Restoration of the Vasopressinergic system

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By

Lynn M. DiBenedetto

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December 1997

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It has been said many times that science cannot be conducted in isolation and so is the case with this dissertation. I have been extremely fortunate to have interacted with so many amazing people during the course of my graduate work. All have contributed in some way to my scientific or personal development. The faculty and staff from the department of Cell Biology with whom I have had the pleasure of working and learning are all very special people. I am particularly indebted to Dr. Susan Gagliardi and Christine Powers for teaching me the finer points of small animal perfusion, electron microscopy, and animal colony maintenance. Anne Gilroy took me under her wing and gave of her time, expertise, and friendship unreservedly. The support and encouragement from many friends has been so greatly appreciated.

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invaluable these past few months as I attempted to finish my research 3,000 miles away from the institution. Without e-mail and the constant patience and hard work of Yvon, I couldn't have done it. I am very grateful particularly knowing how difficult a time this has been for him.

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The continual support and guidance from each committee member, in ways too numerous to mention, is the embodiment of all that is good in science and all that I hope to pass on to the next generation of budding scientists. Thank you so very much.

DEDICATION

TO MY HUSBAND STEPHEN

The commas of the world can't thank you enough for your incredible eye for detail and I
can't thank you enough for all of your love and support.

AND TO PAIGE

Thank you for all the sunshine you bring into my life every day.

ABSTRACT

The hypothalamo-neurohypophysial model has been studied for many years. Of note, when the axons of the magnocellular, peptidergic neurons of the supraoptic nucleus (SON) and paraventricular nucleus (PVN) are transected or crushed, varying degrees of polydipsia and polyuria ensue as the result of measurable losses of vasopressin (AVP) within the organism's circulation. Following insult, these hypothalamic cells show a remarkable capacity to reorganize themselves within the proximal areas of the infundibular stalk and median eminence and form what has come to be known as a new 'mini neural lobe'. While the surviving neurons sprout new projections toward the level of the external zone, vascular hypertrophy is marked throughout the new neurohypophysis and new neurohemal contacts have been identified (at the ultrastructural level) associated with these vessels. In parallel with this vascular hypertrophy is a measurable re-release of vasopressin into the circulation. This new 'mini neural lobe' now has the morphological and physiological appearance of an intact neural lobe and is capable of releasing AVP in response to changes in water balance.

While the ability of these axons to reorganize is more characteristic of the peripheral nervous system (PNS), this model system provides an unique opportunity to study axonal regeneration of the central nervous system (CNS). Not only the mechanisms underlying the restoration of AVP function following axotomy but the extent to which various magnocellular neuron populations are involved in the regenerative process may also be analyzed. Before attempting to identify putative markers associated with this regenerative process, it was necessary to carefully characterize the system following axonal injury. Using Sprague Dawley rats, we repeated previous physiological studies which had examined the intake of water and output of urine following hypophysectomy.

In addition, we also correlated the restoration of water balance with the return of AVP release, as measured by radioimmunoassay. These data defined a temporal framework in which magnocellular AVP regeneration occurs.

As a result of repeating these physiological studies, we noted several inconsistencies between other previously published work. First, the time course of AVP recovery did not agree with other published results, nor did the first appearance of AVP immunoreactivity. We did not observe a complete recovery of water balance as previously reported and the degree of magnocellular death was inconsistent with other reports. In light of these many conflicting observations between several historical reports and our own results, we did a basic physiological re-characterization of the hypothalamo-neurohypophysial system following hypophysectomy. By means of immunohistochemistry, we also demonstrated the re-appearance of AVP within the new 'mini neural lobe' concomitant with the increased appearance of synapsin I, a marker associated with the presence of mature and presumably functioning synapses to be no sooner than 28 days following surgical removal of the hypophysis.

Immunocytochemistry was also used in conjunction with retrograde fluorescent labeling to extend the previous studies and include a 2-D analysis of cell survival throughout the PVN and SON following hypophysectomy or neurohypophysectomy. As reported previously, magnocellular neuronal loss is greater within the SON, particularly the hypophysectomized subject, and less so within the PVN; again with the greater loss in the PVN of the hypophysectomized animal. Based upon our observations and other recent reports, we suggest the possibility that some cells of the hypothalamo-neurohypophysial system or some other extrahypothalamic cell population may be capable of expressing vasopressin in response to neurohypophysectomy. We provide initial evidence that glial cells of the third ventricle may indeed be involved.

Finally, one of the ultimate goals of using this as a model system of CNS regeneration is to understand the underlying mechanisms and components essential to central nervous tissue regeneration. Toward that end I have been involved with the initial studies to optimize an adenovirus delivery system which will be capable of incorporating various putative neurotransmitter and/or peptide anti-sense messages, being injected into the neurohypophysis and transported back into the cells of the hypothalamo-neurohypophysial system. Once these antisense sequences are expressed by the cells following axotomy, the sequence of expression of various proteins in response to injury may be elucidated.

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CHAPTER I

INTRODUCTION

The endeavor to understand the hypothalamo-neurohypophysial (HNP) system and pituitary gland began centuries ago and yet today we are still asking questions and using newer techniques to learn more about this area of the brain which allows us a glimpse into some of the unique characteristics of the central nervous system. This "uninteresting gland" (Anderson and Haymaker, 1969) with its neuronal afferents has proven not only physiologically interesting but it has intrigued many within the last few decades with its unique neural regenerative properties.

While Vesalius "mercilessly" (Anderson and Haymaker, 1969) attacked most of Galen's work, there did seem to be a consensus among the two that the brain excreted waste material (pituita) through the infundibulum into the pituitary. From the pituitary this waste was somehow passed into the nasopharynx and removed from the body as nasal mucus. (As an interesting aside, Vesalius described this pituita as residue left from the ultrarefinement of animal spirit that had reached the brain in the form of vital spirit from the heart.) Until small glands within the nasal mucosa were found in the late 1600's this belief was upheld. Interestingly, two hundred years later Michael Foster wrote in his Textbook of Physiology "with regard to the purposes of the organ [pituitary] as a whole we know absolutely nothing" (1891).

In 1894 Ramon Y Cajal first described fibers that originated in the hypothalamus and terminated in the posterior lobe but not until the mid 1900's did Bargmann show that these same fibers transported neurosecretory material from their cell bodies into the posterior lobe (Bargmann, 1948). The dogma that the pituitary gland presented only minimal interest was changing as more clinical data were gathered. As early as 1881

some physiological function was being associated with the pituitary area. Herman (1881) believed that one of his patients who presented with a "fierce thirst" following a strike to the back of his head had injured the area of the floor of the fourth ventricle. Nothnagel (1921) was the first to suggest that some area of the central nervous system was sensitive to water balance. In addition to the polydipsia which seemed to accompany this type of brain injury, pituitary tumors resulted in the development of diabetes insipidus (Bramwell, 1888 in Anderson and Haymaker, 1969). Diabetes insipidus as we know today, is characterized by a deficiency in the neurosecretory material, antidiuretic hormone, which results in a diminished ability of the kidney to conserve water, hence water loss is excessive and dehydration ensues.

The pituitary gland has an interesting embryological beginning. The anterior portion of the gland is derived from ectodermal tissue (Rathke's pouch) from the primitive mouth cavity and has a characteristic glandular appearance while the posterior portion of the gland is derived from neural tissue extending from the floor of the third ventricle (diencephalon) eventually forming an extension of the central nervous system known as the neurohypophysis. The neurohypophysis may be further divided into three distinct parts: infundibular process (the neural lobe); the infundibular stem (neural stalk); and the median eminence (tuber cinereum)(Moore,1988). Each portion is capable of releasing hormone into the general circulation. The major innervation of the neurohypophysis is supplied primarily by the supraoptic and paraventricular nuclei of the hypothalamus. The structure and ultrastructure of the neurohypophysis have been described in detail (Stutinsky, 1957, Monroe, 1967). Bargmann (1967) described the neurohypophysis as a collection of nerve fibers and their terminal and pre-terminal dilatations, and a rich network of blood vessels with some glial cells scattered in between. The neurohypophysis itself is very similar morphologically to the median eminence, both

having a nervous parenchyma supported by specialized glial cells, or pituicytes. In addition to the rich network of blood vessels, the neurohypophysis has as a characteristic feature extensive perivascular space. Fenestrated capillaries are found within the perivascular space along with collagen and fibroblasts. Fibroblasts may be required for the maintenance of the wide and branching perivascular spaces. The perivascular space is more complex in neurohypophysis than median eminence.

The removal of the pituitary gland, i.e. hypophysectomy, was first used as a model to study acromegaly. It was believed that malfunction of the pituitary gland resulted in this condition. However, when the pituitary glands of dogs were removed via a trans-temporal approach, the animals did not survive more than 24 hours and it was concluded that the gland was essential to life. Using another approach to the pituitary (through the roof of the mouth), however, resulted in dogs that survived up to two years. These animals did not present with symptoms of acromegaly but did show a failure to grow (Aschner 1909 in Anderson and Haymaker, 1969). A modified version of the latter method which approaches the pituitary using a paratracheal approach is one of the two more commonly used techniques today.

By using the newly developed Clarke stereotaxic apparatus on monkeys and cats, Fisher et al. (1938) severed the supraoptico-hypophysial tract as it traveled over the optic chiasm and the result was marked diabetes insipidus. Further study of these animals showed that the SON was completely degenerated, the median eminence reduced in size, and the pars nervosa (neurohypophysis) atrophic. These results were in complete opposition to Bailey's earlier interpretation of his results following trans-temporal hypophysectomy, in which he and Bremer state that disturbance of the innervation to the neural lobe is not the cause for diabetes and caused no symptoms (1921). While this interpretation seems so incredible in light of what is known today, it serves to illustrate

the beginning of a long history of conflicting reports and observations relating to the neurohypophysis, hypophysectomy, and the release of antidiuretic hormone into the general circulation.

In a classic paper, Starling and Verney (1924) demonstrated an antidiuretic effect on isolated kidney preparations from extracts of the pituitary gland. Because this was the first role identified for the extract, the hormone was originally name antidiuretic hormone (ADH). Since then ADH has been shown to have several other effects and its amino acid sequence has been determined. It is a nonapeptide which differs from other analogous peptides in various species by having an arginine in position 8. As a result of the ability of this nonapeptide (known as ADH) to act as a potent vasoconstrictor when released into the general circulation in response to decreases in blood volume, ADH is more commonly referred to as arginine vasopressin (AVP). During the mid 1940's the role of AVP in the maintenance of fluid and electrolyte balance had been acknowledged (Verney 1947), as had its site of synthesis (primarily) within the magnocellular neurons of hypothalamus (Verney 1947), and it's eventual release from the neurohypophysis. Prior to this work, Chambers et al. (1945) demonstrated that the intravenous administration of solutions that increase plasma osmotic pressure in dogs produced a release of AVP from the animals' neurohypophyses. Intracarotid infusions of saline which increased pressure as little as 1.8% resulted in release of measurable amounts of AVP leading Verney (1947) to propose the existence of osmoreceptors within the hypothalamus. Today it is known that there are many other physiological stimuli in addition to hypertonic saline infusions which cause the release of measurable amounts of AVP (Share, 1974).

In addition to our understanding that AVP is synthesized by magnocellular neurons of the hypothalamus and released into the general circulation via the neurohypophysis, the hypothalamo-neurohypophysial system is also known for its

incredible plasticity. Stutinsky was one of the first researchers to interrupt the infundibular stem of rats and observe the restructuring of a miniature neural lobe within the proximal part of the stem (Stutinsky, 1951, 1952). Subsequently, many other species have also been lesioned within the corresponding area with similar results (see Moll, DeWied 1962 paper for review of species.) It has also been demonstrated that neurosecretory hormones are produced and released in animals which have a reorganized neurohypophysis (Lloyd, Pierog 1955, Benson and Cowie 1956, Stutinsky 1957a, Raisman, 1973).

This plastic phenomenon within a portion of the central nervous system is of obvious importance because it provides an accessible model system in which to address many questions about the mechanisms of neuronal regeneration and restoration of biological function. Earlier studies have examined such parameters as water consumption, urine excretion, AVP release in response to hemorrhage, dehydration, plasma osmolarity changes, and time required for neuronal reorganization within the system. As data have accumulated, our understanding of this system has become increasingly complex and several studies have left unresolved questions: to what extent is AVP released following hypophysectomy, how much time is involved in the regenerative process, is recovery partial or complete, what happens to the neurons innervating the injured neurohypophysis, and are uninjured axonal terminals within the median eminence or infundibular stem affected?

Of the studies regarding AVP release following transection or hypophysectomy, regardless of the type of assay used, most concur that it is indeed re-released into the general circulation of these animals. Inconsistencies arise when the amount is quantified or measured in response to various physiological stimuli such as hemorrhage, dehydration, stress, or hypertonic saline injections. In addition to a range of reports

detailing the temporal profile of functional recovery (Lloyd et al., 1955, Moll et al., 1962, Raisman, 1973), the extent of magnocellular neuron loss within the SON and/or PVN has also varied greatly. Following hypophysectomy, reports have ranged anywhere from 50-90% loss of magnocellular neurons in the SON and 10-40% cell loss in the PVN (Raisman, 1973, Rasmussen, 1940). Hence, our understanding of the morphological and functional plasticity of the hypothalamo-neurohypophysial system following injury remains an interesting and fruitful area of study. Indeed, this region of the central nervous system provides a unique opportunity to study the regenerative properties of axotomized neurons.

In the following studies we examined multiple aspects related to the morphological and physiological restoration of the AVP neurosecretory system following the insult of hypophysectomy or neurohypophysectomy. We present a detailed physiological profile of water and urine balance as well as a temporal profile of the restoration of AVP release concomitant with the appearance of synapsin I protein in the newly reorganized "mini neural lobe". Synapsin I protein is expressed within mature and functioning synapses. Also, the system is further described by identifying those vasopressinergic neurons which survive hypophysectomy or neurohypophysectomy. The possibility that other non-vasopressinergic or extrahypothalamic cells may express AVP in response to axotomy of the HNH system is examined.

CHAPTER II

FUNCTIONAL RESTORATION OF THE HYPOTHALAMO-NEUROHYPOPHYSIAL SYSTEM CORRELATES WITH THE RE-APPEARANCE OF SYNAPSIN I IMMUNOREACTIVITY IN THE 'MINI NEURAL LOBE'

INTRODUCTION

Arginine vasopressin is synthesized in magnocellular neurons primarily localized to the supraoptic (SON) and paraventricular (PVN) nuclei of the hypothalamus. These neurons send long axonal processes caudally through the internal zone of the median eminence to terminate in the perivascular spaces of the neurohypophysis making neurohemal contacts with fenestrated capillaries (Scharrer et al., 1954). Arginine vasopressin is transported down these neurosecretory fibers and stored in synaptic vesicles in the nerve terminals (Bargmann et al., 1951). Stimuli such as decreases in blood volume (Wakerly et al., 1975, Poulain et al., 1977) and increases in plasma osmolarity (Arnauld et al., 1974, Haskins et al., 1975, Brimble et al., 1977) activate the magnocellular neurons in this hypothalamo-neurohypophysial (HNP) system to release AVP. The action potential-mediated depolarization of the nerve endings results in the release of AVP from the neurohypophysis into the general circulation (Verney, 1947, Weinstein et al., 1960, Dunn et al., 1973) where it functions primarily as an antidiuretic by decreasing solute-free water clearance in the kidney (Starling et al., 1925).

When AVP neurosecretory nerve endings are destroyed by hypophysectomy or transection there is a loss of antidiuretic function associated with polydipsia and polyuria

(Stutinsky, 1951, Lloyd and Pierog, 1955). The AVP cell bodies in the SON and PVN undergo morphological and neurochemical changes following damage to their neurosecretory nerve endings (Raisman, 1973a). In the weeks following insult, there is a loss in AVP immunoreactivity within the neurohypophysis and median eminence and many of the neurosecretory magnocellular neurons die. However, those neurons that survive reorganize new axonal terminals which reinvest the perivascular space and resume their neurosecretory function (Billenstien and Leveque, 1955, Moll, 1957, Raisman, 1973a). If there is a complete absence of the pituitary gland, as is the case with hypophysectomy, the microvessels of the external zone of the median eminence undergo dramatic angiogenesis and expand into the overlying internal zone (Moll, 1958). This rich capillary plexus provides new vascular surface for the sprouting neurosecretory nerve endings and together they comprise a 'mini neural lobe' from which AVP can be released into the general circulation (Raisman, 1973b).

While it is important to note that the reorganization of magnocellular neurosecretory axons within the hypophysial tract and neurohypophysis are neurohemal specializations and not true neuro-neuronal synapses, work by Raisman et al. (1973b) has shown that the specializations found in the newly reorganizing axon terminals appear to be in every way comparable to true neuro-neuronal synapses. It is most probable that the observed events of reorganization within this neurosecretory system may provide valuable insights into the general mechanics of central nervous system regeneration. Thus, we have chosen the neurohypophysial model system using male, Sprague Dawley rats to examine the temporal profile of one of many neural proteins putatively involved in synaptic plasticity or nerve regrowth. This model system allows one to demonstrate a temporal relationship between re-expressed proteins and reorganized, functioning synaptoid contacts.

Synapsin I represents one of the many neural proteins involved in synaptic plasticity. It is the best characterized member of a family of nerve terminal-specific phosphoproteins thought to play a role in the process of neural transmission. Within the presynaptic nerve terminal, synapsin I is associated with small synaptic vesicles and with specific components of the cytoskeleton (Nestler and Greengard, 1984). The phosphorylation state of synapsin I has been shown to be coupled to the release of neurotransmitter from presynaptic terminals, and the prevailing supposition is that the protein functions as a neuron-specific regulator of the release process. For example, following transection of the perforant pathway to the hippocampus, there is a loss of synapsin I immunoreactivity in the molecular layer of the dentate gyrus (i.e., the terminal zone of perforant pathway fibers) that reappears weeks later (Melloni et al., 1994). It has been postulated that the reappearance of synapsin I protein in the denervated zone signals the restoration of functional synaptic activity. However, in the case of synapsin I and many other proteins of synaptic plasticity and function, it has been difficult to correlate their temporal changes with recovery of distinct, measurable physiological or behavioral functions. Using immunohistochemistry, the present study seeks to correlate the reappearance of AVP neurosecretory function with synapsin I, a known marker of synaptic plasticity.

In the present study, we used measures of water balance and the release of AVP into the general circulation as physiological endpoints marking the functional restoration of the AVP neurosecretory system following hypophysectomy. Specifically, daily water intake and urine output were measured for eight weeks following hypophysectomy. At each week, plasma levels of AVP were determined in response to hemorrhagic-like stress. These physiological endpoints were correlated with morphological changes in the median eminence associated with the loss and subsequent reappearance of AVP and synapsin I immunostaining.

RESULTS

Water and Urine Measurements

Daily water intake and urine output were measured for each animal. Animals were observed for 56 days post hypophysectomy and because there was no significant difference between 28 and 56 days, all data between 28 and 56 days have been omitted from the figures. A two-way factorial ANOVA examining the effect of hypophysectomy on water intake over a 28 day period showed a significant difference between control and experimental animals [$F(1,12)=86.1$, $P<0.001$] (Fig. 1) Within the hypophysectomized group, animals decreased their water intake by approximately 20 to 30% over the observed time but they did not completely recover when compared to age matched control animals. In addition, there was a significant difference between the day 10 post hypophysectomized group and the final day of data collection (56 d) ($P<0.01$). Control animals ($n=10$) showed a mean daily water intake of approximately 10-15 ml/100g over the course of the study. Initially the amount of water consumed by hypophysectomized animals ($n=17$) was approximately 35 ml/100g. By day 21, however, water intake began to decline in the experimental group and by day 28 water consumption was consistently in the range of 20-25 ml/100g body weight for the remainder of the study.

Urine output data paralleled water consumption for both groups. Within the experimental group urine levels decreased by approximately 55- 60% from day 7 to day 56 post-hypophysectomy. Also, urine output was significantly different between hypophysectomized and control groups throughout the study [$F(1,11)= 324.7$, $P<0.001$] (Fig. 2).

FIGURE 1. Daily measurements of water intake of rats following hypophysectomy for control (n=10) and hypophysectomized (n=17) rats normalized to 100g body weight \pm SEM. AVP release between day 2 and 28 post-hypophysectomy, were significantly different. (Student's t-Test $P < 0.01$)

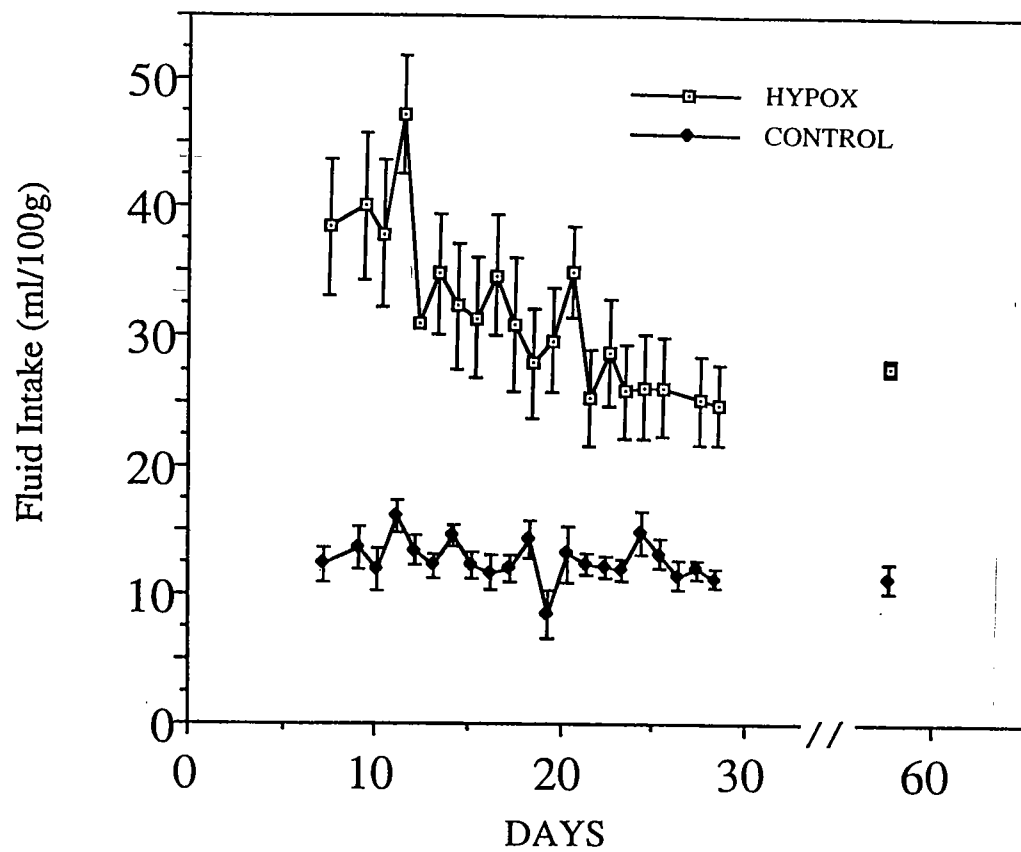
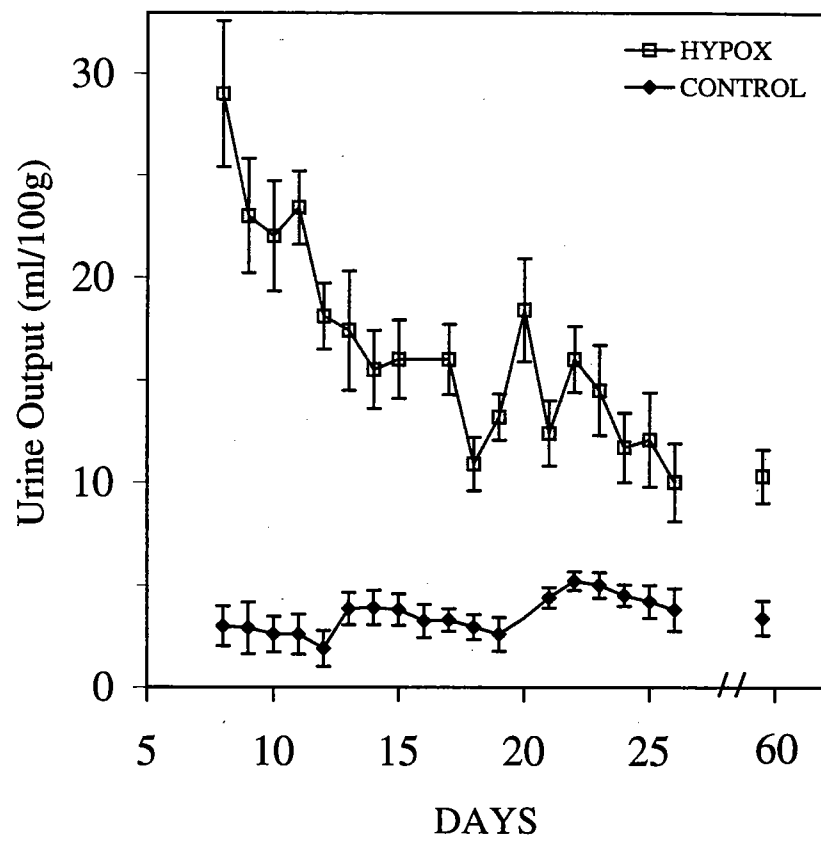


FIGURE 2. Daily measurement of urine excretion by rats following hypophysectomy for control (n=10) and hypophysectomized (n=17) rats normalized to 100g body weight \pm SEM. Note there is a significant difference between experimental animals at day 8 versus day 28 post-hypophysectomy. (Student's t-Test $P < 0.001$)



Arginine Vasopressin Levels

Using radioimmunoassay, plasma AVP levels of control animals ranged between 300 to 1200 pg/ml in response to hemorrhagic-like stress (Fig. 3). A one-way factorial ANOVA revealed a significant difference between experimental and intact animals [$F(5,40)=24.3$, $P<0.001$; see Fig. 3]. *Post-hoc* comparisons showed that day 7 versus day 28 post-hypophysectomy were significantly different (Student-Newman-Keuls <0.05). As expected, there was a dramatic decrease in plasma AVP levels following hypophysectomy. However, 28 days after surgery, animals showed a significant increase in plasma AVP levels as compared to early post hypophysectomy periods in response to an opened thorax and cardiac bleed. These values, though increased markedly from day 7 post-surgery, were still less than 25% of AVP levels for control animals subjected to hemorrhage. This submaximal release of AVP in response to hemorrhagic-like stress persisted through day 56.

Immunocytochemistry

Immunocytochemistry was used to observe the restoration of AVP and synapsin I expression in response to hypophysectomy over time. Sagittal sections through the median eminence in control animals were stained with anti-AVP or anti-synapsin I to establish a baseline of normal immunoreactivity (Figs. 4, 5). The internal zone of the median eminence and external zone are clearly differentiated with AVP immunoreactivity quite robust within the internal zone (Fig. 4). This intense immunostaining reflects AVP within vesicles being transported down large varicose axons to the neurohypophysis. The punctate appearance of AVP within the external zone is presumably due to neurohemal contacts from neuroendocrine cells located throughout the hypothalamus terminating here (Lechan, et al., 1982).

Following hypophysectomy, the majority of neurosecretory axons traveling to the neurohypophysis degenerate and the normal pattern of AVP and synapsin I immunoreactivity in the internal zone of the median eminence (ME) is significantly altered as early as seven days following surgery. At day 7 post-hypophysectomy, the ME is already losing some of its previous organization but it is still visible throughout the internal and external zones (Fig. 4). This persistence of immunoreactivity is thought to be the result of continued transport of neurosecretory material to the cut proximal ends of the vasopressinergic nerve fibers (Raisman, 1973a) where release is no longer possible.

Synapsin I immunoreactivity within the internal zone of an intact rat is virtually absent since the majority of magnocellular neurons of the SON traveling through this region do not form synapses until they reach the neurohypophysis. The external zone, however, reveals punctate synapsin I immunoreactivity which is the result of neurohemal contacts being made by other extrahypothalamic neurons with the vasculature of the portal circulation within the median eminence. Following hypophysectomy, synapsin I immunoreactivity within the internal zone is still quite sparse after seven days (Fig. 5).

By 21 days, post-hypophysectomy, the organization of the internal zone is virtually lost concurrent with the decline of AVP and synapsin I immunoreactivity (Figs. 4, 5). At a higher magnification one can observe an obvious hypertrophy of vasculature in the internal zone (Fig. 6). By 28 days following hypophysectomy, however, both AVP and synapsin I immunoreactivity reappear (Fig. 6) coincident with the increased plasma levels of AVP released during hemorrhagic-like stress. AVP immunoreactivity in the experimental animal is no longer organized in longitudinal fibers within a defined median eminence and synapsin I is observed to be characteristically punctate in nature surrounding new vasculature. Thus, approximately 28 days after the initial insult, the

proximal end of the pituitary stalk and median eminence no longer have an organized zonal appearance.

FIGURE 3. The effect of hemorrhagic-like stress on the release of AVP in normal and hypophysectomized rats of varying post-operative ages (mean \pm SEM) using radioimmunoassay. (Note that three to four mls of blood were collected over a period of two to three minutes.) A one-way factorial ANOVA revealed a significant difference between experimental and intact animals [$F(5,40)=24.3$, $P<0.001$]. *Post-hoc* comparisons showed that day 7 and day 56 post-hypophysectomy were significantly different (Student-Newman-Keuls <0.01).

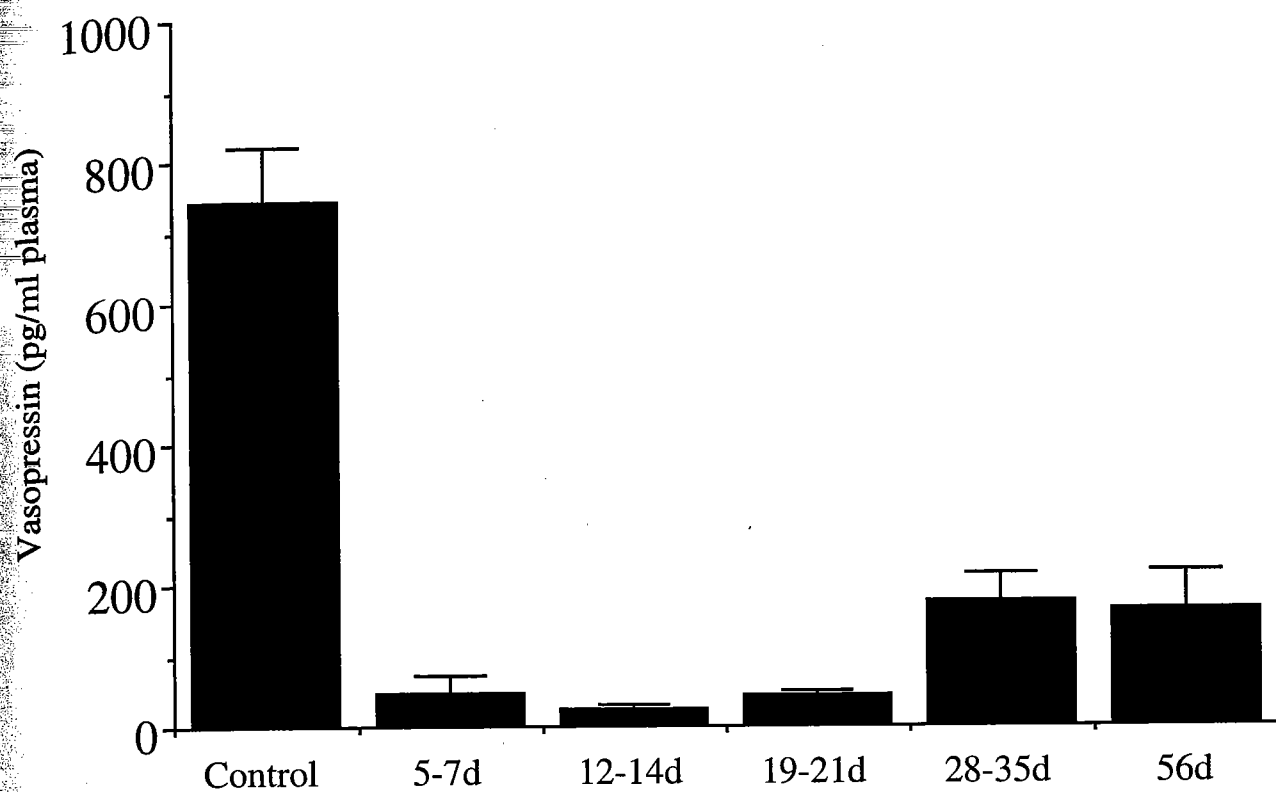


FIGURE 4. An immunohistochemical light micrograph of the median eminence from a control rat (Con) and hypophysectomized (Hypox) rats 7, 21, and 28 days following surgery. Sagittal sections (35 μ m) were stained for AVP. Note the well defined internal (arrows) and external zone (arrowheads) in the control panel. Twenty one days following hypophysectomy, no discernible internal zone pattern remains. By day 28, however, AVP immunostaining is markedly increased throughout the median eminence. Complete immunohistochemical data sets for each time point were obtained from three to five animals. Scale Bar = 50 μ m

Control



Hypox

7d



21d



28d



FIGURE 5. An immunohistochemical light micrograph of synapsin I immunoreactivity within the median eminence. The control panel (Con) shows little immunoreactivity due to the majority of en passant fibers not terminating within the internal zone. Twenty-eight days following hypophysectomy (Hypox), the presence of mature synapses is suggested by punctate immunoreactivity within the newly reorganized area of the internal zone.

Scale Bars = 50 μ m

Con



Hypox

7d



21d



28d



Rather, the entire structure is highly vascular with no apparent compartmentalization of AVP or synapsin I immunoreactive elements. Although there were significant overall changes in the pattern and intensity of AVP and synapsin I immunoreactivity in the internal zone of the median eminence during the period between 28 and 56 days post-hypophysectomy, no comparable changes were observed in the median eminence of sham operated or naive control animals.

Higher magnification study of the same brain sections (Fig. 6) reinforces the finding that AVP and synapsin I immunoreactivity surround many of the newly formed blood vessels within the reorganized 'mini neural lobe'. Whereas little to no punctate synapsin I immunostaining is visible on day 21, seven days later (day 28), punctate synapsin I-specific staining is observed throughout the newly organized neural lobe. Also, AVP immunostaining is much more robust at 28 days than 21 days post-surgery concomitant with the highest plasma levels of AVP release into the general circulation.

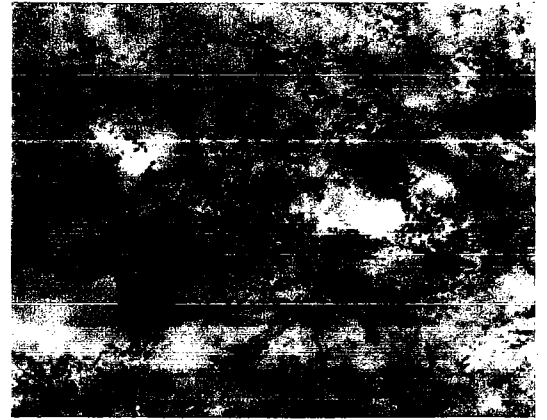
To establish that these changes in synapsin I immunoreactivity were unique to the HNH system and not ubiquitous within the CNS, synapsin I immunoreactivity was measured within the dentate gyrus of intact ($n=3$) and hypophysectomized animals at days 7 ($n=3$) and 28 ($n=4$) post surgery. Table 1 shows no significant difference between synapsin I density within normal animals versus experimental animals or between the experimental animals over time ($P>0.5$).

FIGURE 6. The same light micrographs of figures 4 and 5 at higher magnification (63x oil lens). All micrographs were taken within the area previously defined as the internal zone. AVP is obvious (control) within fibers passing through the internal zone of the median eminence (arrows) and essentially absent after 21 days. By the fourth week, however, the presence of AVP is markedly re-established within the median eminence (28d), notably surrounding the newly formed vasculature (asterisks). Synapsin I immunostaining is also dramatically increased by day 28 as compared to the control panel and earlier periods of surgical recovery.

AVP

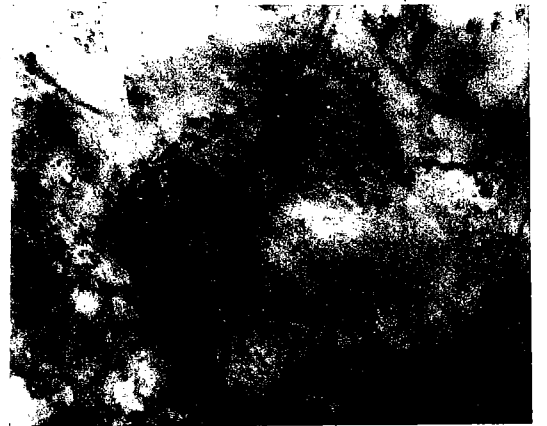
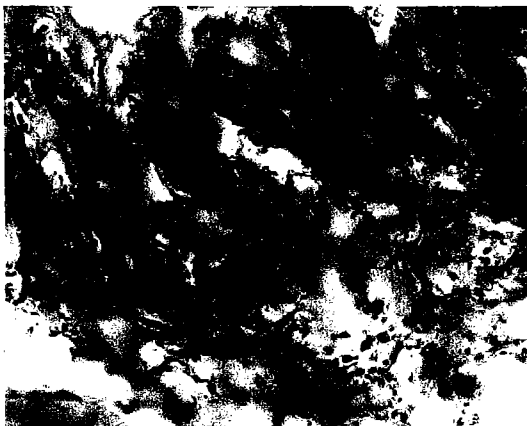
SYN I

Control

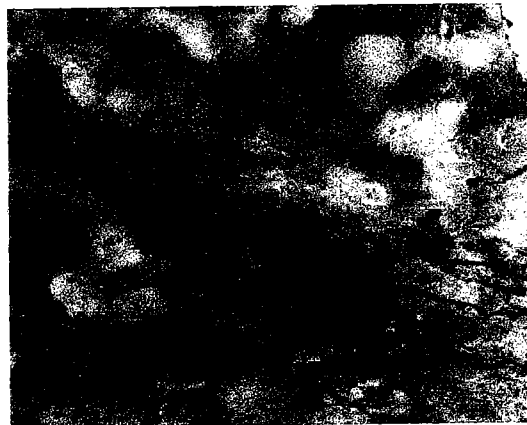


Hypox

7d



21d



28d

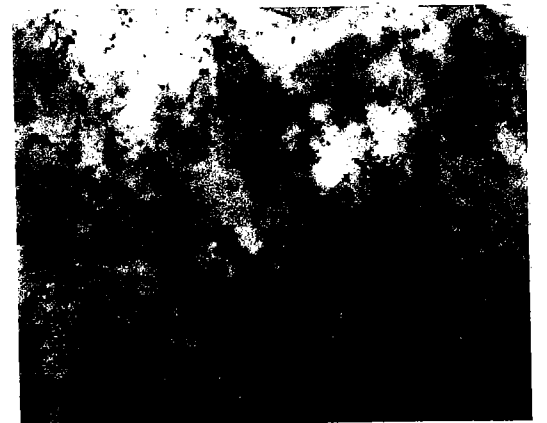
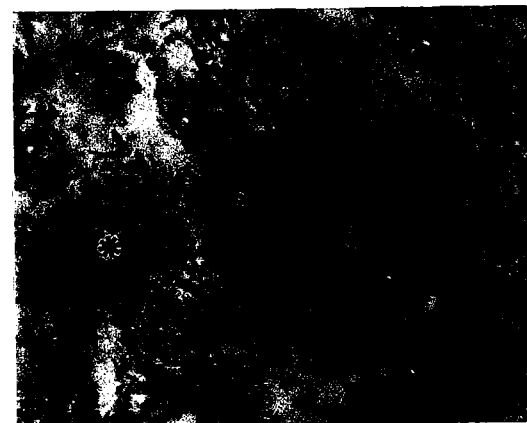


Table 1. Densitometric measurements of synapsin I immunoreactivity within the dentate gyrus of intact and experimental animals. Each measurement is the average of ten individual measurements (ten micrometer spheres) taken within the dentate gyrus of the same animal. Statistical analysis (single factor ANOVA) reveals no significant difference between the control and experimental animals [$F(2,7) = 0.0468$, $P = 0.954$].

	Density	Mean Values
Control	17.29	
(n=3)	19.98	17.8 ± 2.1
	15.96	
Hypox 7d	16.55	
(n=3)	16.95	17.4 ± 1.2
	18.80	
Hypox 28d	15.90	
(n=4)	16.78	17.5 ± 1.5
	17.83	
	19.48	

DISCUSSION

The present study is a further refinement of the experimental model extensively studied by Stutinsky (1951), Moll and DeWied (1962), Raisman (1973a,b) and several others. While Raisman elegantly detailed the morphological events within the median eminence following hypophysectomy, the correlative data attempting to link reorganization of the HNH system with functional release of AVP has been clouded by several varied and conflicting studies. Most studies prior to this have relied on a bio-assay to measure AVP levels in an ethanol anesthetized, water loaded animal (Ginsburg and Heller, 1953, Billenstein and Leveque, 1955, Baratz and Ingraham, 1959, Moll and DeWied, 1962). Although this work has been cited repeatedly in more recent studies, Moll and DeWied (1962) noted that the bio-assay was suspect.

The data presented over the years concerning the duration of polydipsia/polyuria (Laszlo et al., 1966, Moll, 1962), the time frame in which AVP re-appears in the median eminence, (Mirsky et al., 1954, Lloyd and Pierog, 1955, Benson and Cowie, 1956, Raisman, 1973a), the conditions under which AVP is released from the newly reorganized system (Billenstein and Leveque, 1955, Moll and DeWied, 1962), and nerve regeneration within this system itself have also been many and varied. Our present data using improved immunohistochemical procedures and a highly specific RIA corroborate only some of the earlier reports. We too have demonstrated that hypophysectomy causes a dramatic increase in urine output and water intake followed by a partial recovery of water balance over a three to four week period. However, using water balance as a measure of functional restoration of neurosecretory activity is imprecise because the urine output and water intake never return to basal values, despite reports that the osmotic release of AVP in the reorganized mini neural lobe returns to normal levels several weeks after injury (Lloyd et al., 1955, Benson and Cowie, 1956). Also, as observed in figures 1

and 2, it appears as though hypophysectomized animals begin to recover some ability to regulate water and urine balance almost immediately after surgical intervention. Several explanations may account for this observed phenomenon. In the early days following removal of the hypophysis, AVP is still being synthesized in the magnocellular neurons and transported toward the damaged axon terminals. Leakage within this area probably contributes to an observable antidiuretic effect as evidenced in water and urine collection data. Raisman (1973) has reported that this continued synthesis by reorganizing neurons ends approximately by day 10 as dense AVP immunoreactivity is no longer present at these terminal ends. This corresponds to a slight increase in water consumption during that time. Before the newly reorganized vasopressinergic system is functional, continued decreases in urine and water measurements may in fact be the result of an altered sensitivity of kidney tubules in response to lowered levels of AVP in the circulation. One explanation of how the kidney might alter its sensitivity has been proposed by Balement (1986). Balement suggests that variation in the ratio between OXY and AVP hormone may play a role in modulating renal handling, of sodium, hence water/urine inputs and outputs would subsequently be effected. Further, he has shown that OXY and AVP act synergistically to regulate sodium excretion. This work serves to strengthen the argument that physiological measurements of water and urine alone are inadequate to describe the re-release of AVP into the general circulation following neuronal reorganization.

To re-examine the time necessary for the neuroanatomical and functional restoration of the AVP neurosecretory activity, we challenged the system with hemorrhagic-like stress, measured plasma AVP levels by RIA, and correlated AVP release with the concomitant appearance of both immunoreactive AVP and synapsin I in the reorganized median eminence. This approach provided neuroanatomical and

biological data defining a window between 21 and 28 days following hypophysectomy as the period of functional restoration of AVP neurosecretory activity.

It has been reported that neurohypophysectomized and hypophysectomized animals can follow moderate dehydration stress and osmotic challenge by releasing levels of AVP comparable to control animals (Lloyd et al., 1955); however, the ability to respond to hemorrhagic-like stress is compromised. Indeed, a later study by Moll and De Wied (1962) failed to detect any release of AVP into the circulation of neurohypophysectomized rats in response to hemorrhage. It is quite possible that AVP levels were undetected due to a technical problem. During the late 1950's the standard method to measure AVP in plasma involved measuring the antidiuretic activity of plasma samples in a water loaded, ethanol anesthetized rat. These animals showed marked diuresis due to the inhibition of endogenous AVP release. This method has subsequently been shown to be a sub-optimal way in which to measure AVP. Radioimmunoassay is much more sensitive and is the preferred method today for detecting AVP in the picomolar range.

More recently Dohanics et al. (1992) challenged stalk-compressed animals (at day 21 post compression) with hypertonic saline infusions and were also unable to elicit a release of AVP in response to osmotic challenge. Assuming the compression model is synonymous with neurohypophysectomy, it is our contention that no AVP was detected by this group because at day 21 post injury the neurosecretory system has not fully reorganized, hence is not yet releasing any AVP into the general circulation.

Here we report the ability to detect a significant release of AVP, approximately 25% of control values, 28 days post-hypophysectomy in response to hemorrhagic-like stress. AVP may not have been detected in the stalk-compressed animals following osmotic challenge (Dohanics et al., 1992) because the neurosecretory system was still

remodeling itself; recall that synapsin I was observed at day 28 and not day 21. We also attempted to elicit a release of AVP following a mild to moderate osmotic stress using mannitol but we were also unable to detect any appreciable levels of AVP in the plasma (unpublished observation). However, for this study we were interested primarily in defining the period when newly reorganized terminals can release AVP. Hence, we chose to use a more robust stimulus for release- hemorrhagy.

Our data show clearly that release of AVP in response to hemorrhagy-like stress follows the recovery of AVP immunostaining in the new 'mini neural lobe'. Indeed, by day 28 post-hypophysectomy, the pattern of AVP immunostaining was markedly more organized and intense than at earlier time points (Fig. 6). Examining the immunoreactivity of AVP in the reorganizing mini-neural lobe from day 7 post surgery until day 28 revealed an abundance of immunoreactivity within the median eminence 7 days following hypophysectomy. This is the obstruction phase (Raisman, 1973b). Neurosecretory cells are continuing to produce and transport AVP to the terminal ends resulting in a build-up of neurosecretory material within the median eminence which cannot be released. By the 14th to 21st day there is a marked increase in vascularization but little to no AVP immunoreactivity within the internal zone. During this destruction phase as described by Raisman, damaged axons are either cleared away by macrophages or reorganized within the new mini-neural lobe and median eminence. By day 28 AVP immunoreactivity has reappeared within the area of the former internal zone, in parallel with AVP release into the blood.

Based on the observation that AVP immunoreactivity and AVP plasma levels are restored at approximately the same time, it is argued that new neurohemal contacts have been established as a result of reorganization of the new 'mini neural lobe' (Moll, 1957, Raisman, 1973b). It is logical to conclude that new, functional synaptoid contacts have

been formed which allow for this release of AVP into the general circulation. Therefore we examined the temporal expression of the nerve terminal-specific protein synapsin I in this brain region. Synapsin I is part of a family of four closely related neuronal phosphoproteins that are localized at presynaptic terminals (synapsin Ia and Ib, and synapsin IIa and IIb). Synapsin in its various isoforms is found in virtually all nerve cells (Sudhof et al., 1989) including the AVP-containing, magnocellular neurons of interest in this study. Synapsin in all its isoforms, comprises approximately 0.6% of total brain protein (Benfenati et al., 1989). They have been implicated in both the regulation of neurotransmitter release and in synapse formation (Han et al., 1991). In addition, recent studies have suggested that the appearance of detectable levels of synapsin I protein in developing and sprouting synapses coincides with secretion by those synapses (Melloni et al., 1994). Thus it is logical to consider synapsin I as a marker of functional axon terminals in this model.

Before and immediately following hypophysectomy there is little synapsin I-like immunoreactivity found within the internal zone of the median eminence. Within 28 days following hypophysectomy, however, there appears a marked increase in punctate synapsin I immunoreactivity within the area previously described as the internal zone (Fig. 6). As the new 'mini neural lobe' organizes, it is morphologically evident that the once clearly distinguishable internal and external zones are no longer discernible, and that new neurohemal contacts are established throughout the median eminence. This time frame of synapsin I reappearance in the median eminence coincides with the recovery of hemorrhagy-induced AVP release into the general circulation. Thus, these data suggest that the appearance of increased levels of synapsin I protein in regenerating synaptic terminals of the new mini neural lobe coincides with the secretion by those synapses.

While these data are compelling, quantitation of synapsin I messenger RNA using *in situ* hybridization will strengthen the proposed time course involved in the re-release of AVP following hypophysectomy as well as verify the presence of newly matured synapses within the 'mini neural lobe'. While directly measuring synaptic function was not within the scope of this dissertation, such measurements would also greatly support the hypothesis that AVP is indeed released from these newly formed synapsin I immunopositive terminals.

Synapsin I may be only one of several other markers of synaptic plasticity essential in the restoration of neural lobe function. Initial immunohistochemical and Western analysis work on several putative peptides such as SCG-10 (superior cervical ganglion), SNAP-25 (synaptosomal associated protein), and GAP-43 (growth associated protein) was begun, however those results were not presented here as they were either inconclusive or of no interest. SCG-10 and SNAP-25 do not seem to be involved in the reorganization of the new 'mini neural lobe' at the time points which were analyzed here and GAP-43 yielded conflicting results which will be require further investigation. It still remains most probable that other markers may play some key roles in this system.

The hypothalamo-neurohypophysial system lends itself quite readily to the study of synaptic reorganization within the nervous system for several reasons. It is an easily definable region of the central nervous system, the magnocellular neurons of the PVN and SON are easily identified, and the neurohypophysis of the pituitary is almost exclusively innervated by fibers from the SON and PVN. Functionally, the neurohypophysial tract is also ideal for such regenerative studies. Unlike the hippocampus, for example, in which much work has been done to understand the peptides and neurotransmitters involved in reactive synaptogenesis, the physiological endpoint or function of the hippocampus is complex and difficult to demonstrate. In contrast, the

temporal pattern of functional restoration of the AVP neurosecretory system is readily identified by measuring the release of neuropeptide into the circulation.

The proteins, peptides, and neurotransmitters involved in this regenerative process are only now being elucidated. The regeneration of damaged axons may well involve the re-expression of cellular growth or neurite extension proteins (Miller et al., 1989, Villar et al., 1994, Pate-Skene, 1989, Cotman et al., 1984) which are expressed during normal development and subsequently down-regulated as the organism matures. Hence, the temporal expression of several peptides and non-peptide putative neurotransmitters have been described using the hypophysectomy model (Villar et al., 1994) but as yet the functional role for many of these peptides has not been directly linked to a physiological function.

MATERIALS AND METHODS

Male rats (150-175g) were hypophysectomized by the vendor (Harlan Sprague Dawley Laboratories, Indianapolis, IN) and shipped four days later to the laboratory along with age-matched controls and sham operated animals. Upon arrival, animals were placed into individual metabolism cages where they were maintained on a normal 12:12 light:dark cycle with standard laboratory rat chow and water ad libitum. The hypophysectomized animals were given a 5% sucrose solution for two days upon arrival as recommended by the vendor. Daily measurements were obtained for water intake and urine output. All fluid measurements were normalized to body weight to account for the marked difference in body size of the control animals versus the much smaller hypophysectomized animals. Animals were sacrificed on days 7, 14, 21, 28, or 56 post hypophysectomy to measure AVP plasma levels. At least 7 hypophysectomized animals were available for radioimmunoassay in each of the survival groups with age matched control animals.

Tissue Fixation

Animals whose brains would be used for AVP immunocytochemistry were perfused transcardially while those brains used for synapsin I immunostaining were immersed directly into a solution of acrolein. In both cases, rats were anesthetized with sodium pentobarbital (50 mg/kg). For transcardial perfusion, one ml of heparinized saline (1000 units) was injected into the left ventricle followed by saline containing 1.5% sodium nitrite, and then a 2.5% acrolein (Aldrich Inc., Milwaukee, WI) in 4% paraformaldehyde in 0.1 M potassium phosphate buffered saline (KPBS), pH 6.8. In the case of immersion fixation, brains were removed and placed into a solution of 10% acrolein in KPBS, pH 7.4 for a total of six hours with one change of fresh fixative after

three hours. After both types of fixation, the brains were immersed overnight in a solution of 25% sucrose in KPBS. All brains were then cut into 30-35 μ m thick sections with a freezing microtome. Tissue sections were stored in cryoprotectant solution (Watson et al., 1986) at -20 °C until they were processed for immunocytochemistry. At least 4 animals for each time point were immunohistochemically processed and analyzed.

Radioimmunoassay (RIA)

Blood samples were drawn from the left ventricle into heparinized tubes just prior to fixing the brains as described above. This procedure was chosen for AVP-RIA because opening the chest cavity results in a decrease in blood pressure due to diminished venous return and cardiac output. The fall in blood pressure stimulates aortic and carotid baroreceptors promoting AVP release from the neurohypophysis. In addition, exposing the thoracic vasculature and heart to the collapsing atmospheric pressure stimulates low pressure baroreceptors in the large veins and the right atrium further driving AVP release (Share, 1974). Therefore, collecting blood from the left ventricle in an open thorax mimics hemorrhagic stress and promotes a massive release of AVP into the general circulation as previously noted (Ferris et al., 1992).

The blood samples were placed on ice and spun to separate plasma. Heparinized plasma samples were stored at -70 °C until assayed. Samples were assayed as previously described (Ferris et al., 1992). Briefly, one ml aliquots of the plasma were run through a C-18 SEP-PAK (Millipore Waters Associates, Milford, MA) to remove large proteins and salts. Two ml of 70% acetonitrile in 0.1% trifluoroacetic acid was used to elute the smaller proteins from the column. Samples were lyophilized overnight and reconstituted in one ml of assay buffer (0.15 M NaCl, 0.05 M NaH₂PO₄, 0.02 M Na₂HPO₄, 0.1% gelatin, 0.02% NaN₃ at pH 7.6). Duplicate samples were run in 12 X 75 ml plastic tubes.

Synthetic, iodinated AVP was purchased from New England Nuclear (Boston, MA) and AVP antisera was obtained from Calbiochem-Behring, CA (lot No. 393079). The antisera has less than 1% cross reactivity with arginine vasotocin, oxytocin, and Lys 8-vasopressin. The antisera was used at a final volume of ca. 1:300,000 in an assay volume of 500 μ l. The iodinated AVP antibody and unknown samples were incubated for 48-72 hr at 4 °C. Free iodinated AVP was separated from bound by adding one ml of a charcoal (2.5%) and Dextran T-70 (0.25%) to each assay tube and spinning for 10 minutes at 4 °C and 4,000 g. The supernatant containing the bound fraction was poured off and counted in a multiwell gamma counter. The sensitivity of the assay was between 0.5 and 1 pg as defined by the 90% maximum binding or 6 to 8 pg as defined by the 50% maximum binding. The intra-assay coefficient of variability was 6.5% and the inter-assay coefficient of variability was 7.3%. The recovery of synthetic AVP (5, 20, 100 pg) initially added to one ml of water and subsequently handled like the plasma samples, ranged between 62% and 76%.

Immunocytochemistry

Brains processed for AVP immunoreactivity involved a procedure described elsewhere and modified slightly (23). Sections were pre-incubated in 20% normal goat serum (NGS), 0.3% Triton-X 100, 3% hydrogen peroxide, and Tris-buffered saline (TBS), pH 7.6, at room temperature (RT) for 30 min. Next, tissue was incubated in primary antisera of AVP 1:16,000 (ICN, Costa Mesa, CA) for 1 hr at 37 °C followed by three - five minute washes in rinse buffer (2% NGS, 3% Triton-X, 95% TBS). The secondary antibody, biotinylated goat anti-rabbit IgG (Vector Laboratories) was used at 0.005% in wash buffer for 45 min at RT. After a series of washes in rinse buffer, sections were incubated with a rabbit ABC Elite kit (Vector Laboratories) and stained with

diaminobenzidine (DAB) (25% DAB, 3% H₂O₂ in TBS, pH 7.6) for 5-10 min at RT. Sections were rinsed, mounted on gelatin coated slides, dehydrated in a series of alcohols, and cover-slipped using Permount (Fisher Scientific). Approximately 30 experimental animals and 20 control animals were processed immunohistochemically for AVP or synapsin I but because the brains were cut in the sagittal plane, several median eminences could not be completely analyzed, therefore a minimum of three animals were analyzed at each time point.

The immunocytochemical procedure used to stain synapsin I terminals within the median eminence was similar to that described for AVP. Tissue sections were incubated in primary rabbit anti-synapsin I serum (1:2000) at 37 °C for 1 hr. The polyclonal anti-synapsin I antiserum used in this study has been previously characterized (Melloni et al., 1993, 1994) and was the kind gift of Dr. Louis DeGenarro. Following the primary incubation, sections were incubated for 45 minutes in biotinylated goat anti-rabbit antisera (Vector Laboratories). After three - five minute washes in rinse buffer, sections were incubated for 45 min using the ABC Elite kit (Vector Laboratories). Sections were washed in TBS and exposed to DAB for 5-10 min, rinsed, then mounted on slides. Control sections included intact and hypophysectomized rats in which the primary antibody, the secondary antibody, or both antibodies were omitted.

The dentate gyrus of both intact (n=3) and hypophysectomized (n=3) rats was also analyzed to demonstrate specific changes in synaptic density within the median eminence. Densitometric analysis of the dentate gyrus is the result of quantification using NIH Image Software (v. 1.56, Bethesda, MD) and gray level thresholding (Shipley, 1989). The peaks of the histograms of the distribution of labeling density were adjusted for each image to the same grayscale value. The results are expressed as the area (in square micrometer) containing immunoreactive signal within standard surfaces (circles of

10 micrometer diameter). Approximately 10 measures were taken for each image. A one-way ANOVA was also used to analyze these results (Table 1).

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CHAPTER III

MAGNOCELLULAR VASOPRESSIN NEURONS: AN ANALYSIS OF CELL SURVIVAL FOLLOWING NEUROHYPOPHYSECTOMY

INTRODUCTION

The neurohypophysial system, with its associated neurons, has long been studied using Golgi (Ramon y Cajal, 1911), silver (Romeis, 1940), and neurosecretory stains (Bargmann 1949). It is only since the development of immunohistochemical methods that we have been able to identify peptides produced by individual neurons (Swabb 1975 a,b, Vandesande and Dierickx, 1975, Sofroniew, 1979). While the use of these techniques has answered several long standing questions, and greatly expanded our understanding about the distribution, projections, and functions of neurons such as the vasopressinergic magnocellular neurons of the SON and PVN, many questions remain regarding the neurohypophysial system.

Early observations by several investigators have described the loss of AVP immunostainable material within the SON, PVN, neurohypophysis, and/or median eminence in response to hypophysectomy. It is clear that cell loss within the SON is dramatic with losses up to 90% (Stutinsky, 1957, Benson et al., 1956). The animals remain quite sick over time, primarily resulting from the loss of the anterior pituitary hormones. As well as presenting with the typical symptoms of diabetes insipidus, hypophysectomized animals are listless and fail to grow. Loss of anterior pituitary hormones, specifically ACTH, elicits AVP gene expression in CRF positive parvocellular neurons in the PVN (Sawchenko, 1984). Hence, in hypophysectomized animals it is not

clear whether changes in AVP gene expression or loss of AVP neurons are due to damage of the AVP neurosecretory system alone or secondary to a more global change in the endocrine milieu and health of the animal. Obviously surgical removal of the neurohypophysis exclusively would provide a cleaner model system for studying the response of the neurosecretory system to injury than total extraction of the pituitary.

One aim of these studies was to compare AVP cell survival in hypophysectomized versus neurohypophysectomized animals. This was done with camera lucida drawings through the SON and PVN of intact, hypophysectomized and neurohypophysectomized animals. Two-dimensional maps were constructed from these drawings and used to compare cell populations between each group of animals.

Another aim of these studies was to examine the compensatory activity of the magnocellular neurosecretory system which may play a part in the restoration of AVP function. The magnocellular neurosecretory system has long been known for its compensatory activity. Moll and DeWied (1962) showed that severed neurosecretory axons sprout to form new neurohemal contacts at the proximal end of the transected infundibulum and Raisman et al. (1973) showed the same sprouting within the external zone of the median eminence. The regenerative ability of magnocellular neurons has been demonstrated in a number of situations: across a severed infundibulum stalk, penetration of intrahypothalamic neural lobe tissue, and into ventral leptomeningeal tissue (Adams et al., 1969, Dellman et al., 1987, Dellman et al., 1988). Watt and co-workers have also demonstrated the ability of uninjured, contralateral magnocellular efferents to undergo a compensatory sprouting as a result of partial denervation of the neural lobe. Hence the AVP neurosecretory system can recover following injury and loss of vasopressinergic cells. Perhaps other neuronal phenotypes in the hypothalamus may start to express AVP *de novo* to compensate for the dramatic changes in fluid in

homeostasis To answer this question, one must be able to differentiate between cells which originally expressed AVP in a normal animal and any new cells which may be expressing AVP following axotomy. This may be done by injecting a retrograde tracer into the neurohypophysis and back-labeling all cells which project to the neurohypophysis, including the magnocellular vasopressinergic population. Then, following removal of the neurohypophysis and subsequent staining for AVP any new AVP synthesizing cells, i.e. those cells which have no retrograde tracer within them, can be identified.

We chose to use the retrograde tracer, fluorogold, for these studies of AVP expression in surviving magnocellular populations for several reasons. It is readily compatible with conventional immunohistochemistry (Ju et al., 1989) and in situ hybridization analysis (Burgunder and Young, 1988). Fluorogold is readily taken up by nerve terminals but not by fibers of passage, and has been shown to remain viable up to 2 months (Schmued et al., 1986). While horseradish peroxidase (HRP) has been extensively used to trace a wide variety of neuronal connections, it has been reported that HRP begins to degrade as early as day 4 following injection, and all enzymatic activity is completely gone by day eight (Price and Fisher 1978). Because of the duration of this study, a more stable retrograde label such as fluorogold was required.

The present study was designed to illustrate the neurons of the HNH system which project to the neural lobe and to identify those vasopressinergic neurons which survive following axotomy and subsequent restoration of the HNH system. By using immunohistochemistry in conjunction with a retrograde label, it is possible to identify all remaining cells which projected to the neurohypophysis, as well as any new AVP-synthesizing cells resulting from neurohypophysectomy. The observations made here support the hypotheses that : 1. all SON-AVP positive cells project to the neural lobe

(Kelly et al., 1980, Sofroniew, 1983, Sherlock et al., 1975), and 2. the loss of supraoptic neurons may range from 50-90%, accounting for individual animal variation and type of surgical intervention. In addition, we find no reproducible evidence for the significant presence of a *de novo* population of cells within the hypothalamus which synthesizes AVP in response to neurohypophysectomy. However, there may exist small populations of extrahypothalamic neurons which are affected by perturbations of the hypothalamo-neurohypophysial system.

RESULTS

Camera Lucida

Camera Lucida drawings through the SON and PVN of control, hypophysectomized (hypox), and neurohypophysectomized (neurohypox) animals (Figs. 7 and 8) confirm that loss of magnocellular AVP-positive neurons is indeed more dramatic in the hypophysectomized animal model (Fig 7B0-4) than in the neurohypox model (fig. 7C0-4) or control animals (A0-4) The PVN drawings show a moderate loss of AVP immunoreactivity within the posterior magnocellular (pm) part of the PVN for both hypox and neurohypox animals (Fig 8B2-3,C2-3), while immunoreactivity for AVP is markedly increased within the medial parvocellular (mp) portion of the PVN in the hypox animal. (Note: The PVN shall be discussed using a simplified description in which the PVN consists of three dense clusters of magnocellular neurons embedded in a larger shell of parvocellular neurons which consists of five components (Swanson et al., 1980)).

Representative photomicrographs for normal, hypophysectomized, and neurohypophysectomized animals detail the classical middle portion of the SON (Fig. 9) and PVN (Fig. 10) (Krieg et al., 1932, Swanson et al., 1980). The SON images confirm the marked cell loss in the hypophysectomized animal and the moderate cell loss following neurohypophysectomy as compared to control magnocellular numbers. In figure 10 the notable increase in AVP staining within the mp area of the hypox animal is presumably due to loss of negative feedback in the HPA axis and up regulation of AVP expression within corticotrophin releasing hormone (CRH) parvocellular neurons. Unlike the hypox model, the neurohypox model has very little immunoreactivity within the parvocellular area and despite the obvious loss of vasopressinergic neurons in the SON (Fig. 9) and PVN (Fig. 10), it appears more similar to the control animal.

Observation throughout the hypothalamic area and camera lucida drawings revealed no new population of cells which were AVP immunopositive after restoration of the injured vasopressinergic system.

Fluorescent Microscopy

Analysis at the light microscopic level showed that virtually all neurons of the SON which were labeled with fluorogold prior to neurohypophysectomy were also labeled with AVP 28 days post-neurohypophysectomy (Fig. 11). This colocalization confirms previous reports which claimed that all magnocellular neurons of the SON project to the neurohypophysis. In many of the labeled cells the fluorogold labeling was very granular within the cytoplasm (arrows). This most probably is due to degradation of the retrograde tracer within lysosomes of the neurosecretory cells 28 days after injection. The medial portion of the PVN also revealed a majority of magnocellular neurons which co-labeled for fluorogold as well as AVP, again supporting the claim that AVP cells of the posterior magnocellular area of the PVN project to the neurohypophysis (Fig. 12). AVP positive cells located in the more caudal regions of the PVN have previously been shown to project to various areas of the CNS such as the brainstem and spinal cord. Observations using an ultraviolet light filter to illuminate the injected fluorogold solution confirmed that the fluorogold injected directly into the neurohypophysis was confined to only the nuclei of the HNH system. No fluorogold immunoreactivity was observed in areas such as mammillary nuclei, habenula, or suprachiasmatic nucleus which have no efferent connections to the neurohypophysis.

AVP and OXY Immunofluorescent Histochemistry

Double immunofluorescent histochemistry using monoclonal antisera to OXY and polyclonal sera to AVP (Fig. 13) revealed a very small population of cells within both the SON and PVN (posterior portion) which appeared to simultaneously express both peptides (n=2). Further, analysis of extrahypothalamic areas of the brain revealed distinct co-expression of AVP and OXY within the retrochiasmatic area (Fig. 14). No double staining was observed within the SCN (supraoptic chiasm). Experimental animals which were stained with pre-absorbed antisera to either AVP or OXY and did not exhibit any co-labeled cells. Control animals also showed no simultaneous labeling of AVP and OXY.

FIGURE 7. Schematic drawings of five representative levels through the SON showing the distribution of AVP immunoreactive magnocellular neurons in control (A-A4), hypox (B-B4), and neurohypox (C-C4) animals. The figures are representative of 3-5 animals (300-350g) for each group, 28 days post surgery. oc=optic chiasm, SON=supraoptic nucleus, III= third ventricle

A

III

SON

oc

B

A₁

B₁

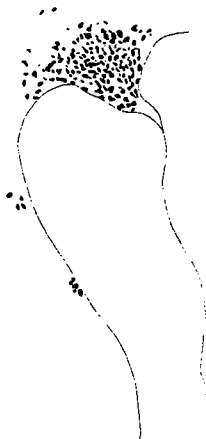
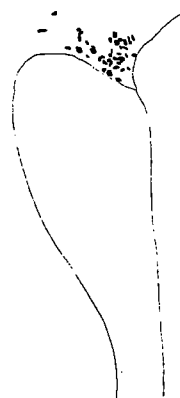
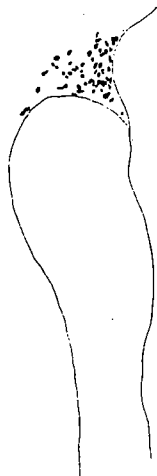
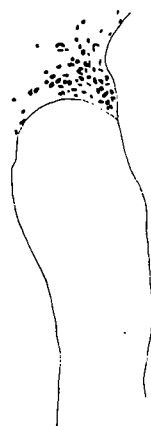
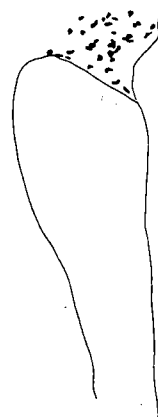
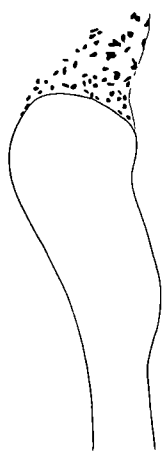
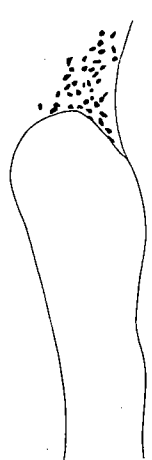
A₂

B₂

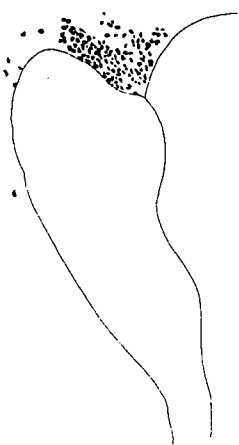
C₁

C₂

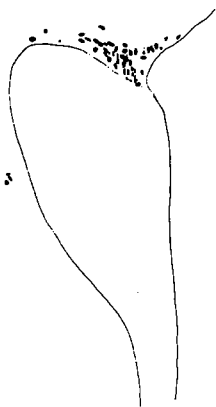
C



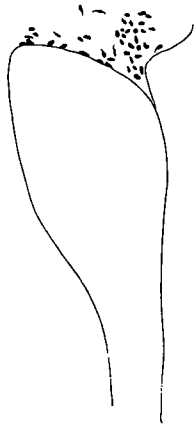
A₃



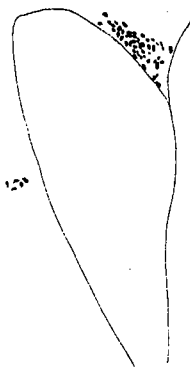
B₃



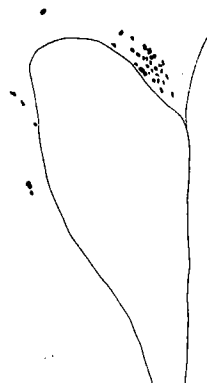
C₃



A₄



B₄



C₄

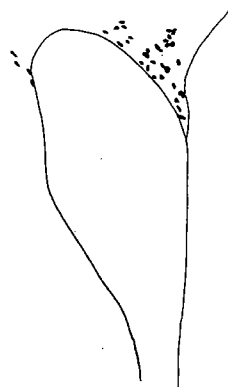


FIGURE 8. Schematic drawings of five representative levels through parts of the PVN showing the distribution of AVP in control (A-A4), hypox (B-B4), and neurohypox (C-C4) animals. Enclosed area (dashed lines) outlines the posterior magnocellular area (pm) of the PVN. All animals (300-350g) were analyzed 28 days post surgery.

mp= medial parvocellular area, pm=posterior magnocellular area, III=third ventricle

A



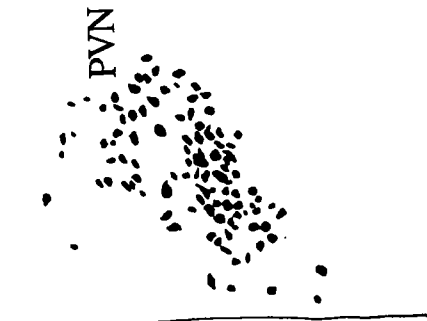
B



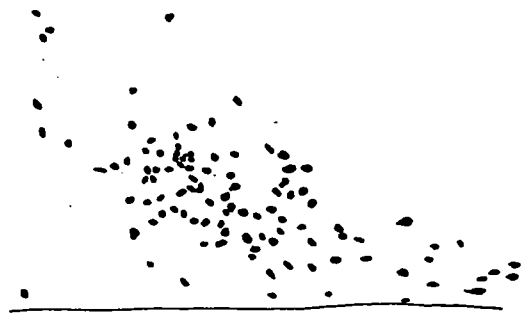
C



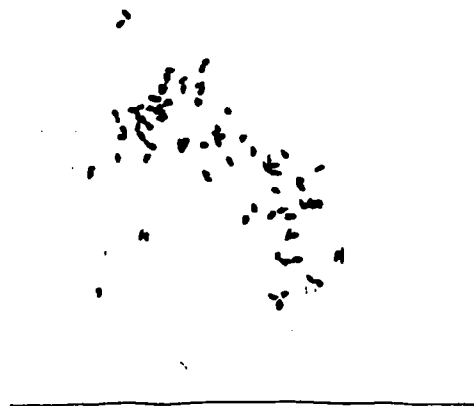
A₁



B₁



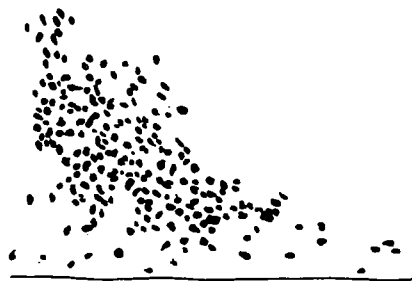
C₁



A₂



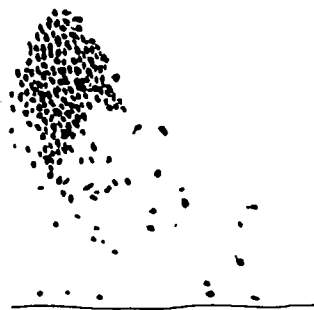
B₂



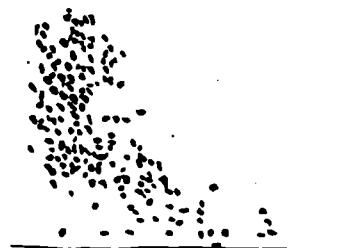
C₂



A₃



B₃



C₃



A₄



B₄



C₄



FIGURE 9. Light photomicrograph (10x) of the middle portion of the SON for control (A), hypox (B), and neurohypox (C) animals. Images represent a total of three animals for each group and were contrast adjusted using Adobe Photoshop. Panel B represents a marked loss of magnocellular neurons following hypophysectomy while AVP cell loss in panel C (neurohypophysectomy) is less dramatic. All sections 35 μ m, coronal plane.

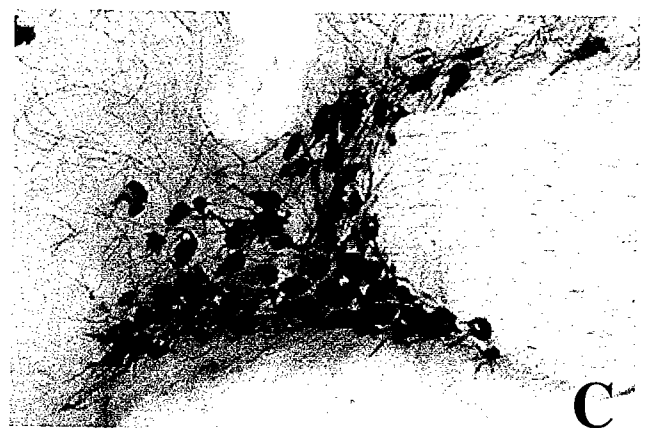
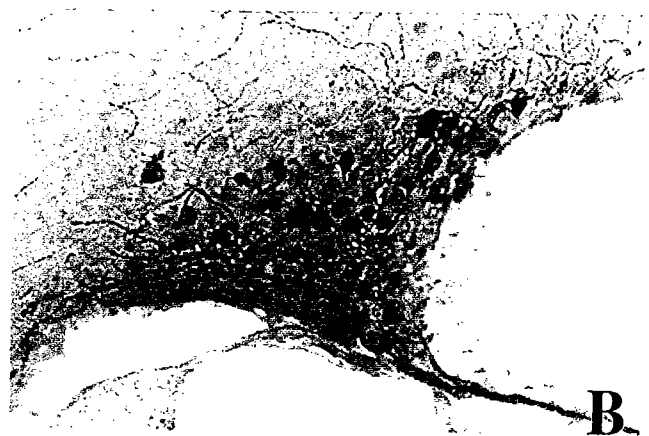
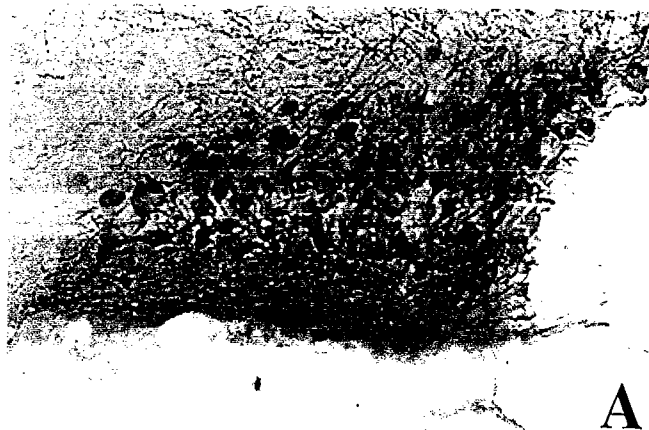


FIGURE 10. Light photomicrograph of the middle portion of the posterior magnocellular area within the PVN (20x). Control (A), hypox (B), and neurohypox (C) animals were stained with AVP antisera. All sections are in the coronal plane and were cut at 35 μ m. Images represent at least three animals for each group. Images were contrast adjusted using Adobe Photoshop.

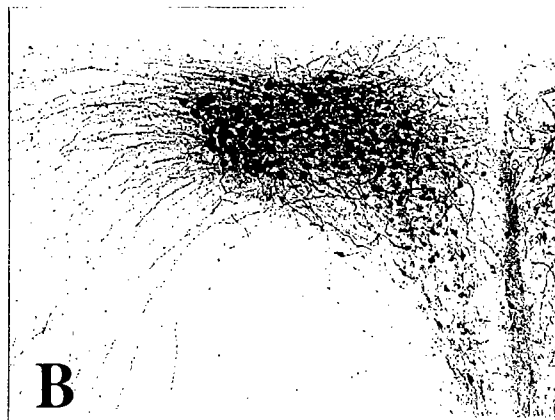
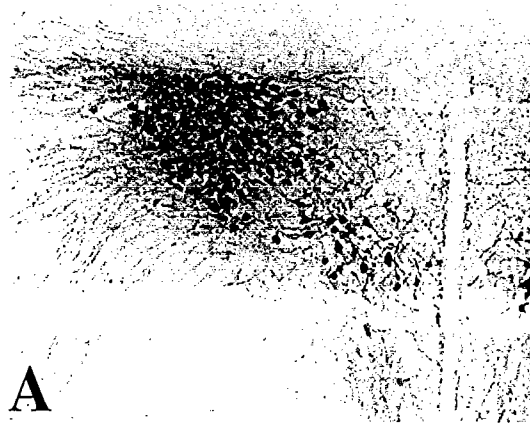


FIGURE 11. Fluorescent photomicrograph (20x) of the SON co-labeled 28 days post-neurohypophysectomy for AVP and fluorogold simultaneously. Fluorogold labeling (panel B) is present in all AVP labeled cells (panel A), see asterisks . The granular appearance within the cytoplasm of some of the fluorogold positive cells may indicate lysosomal degradation (arrows).

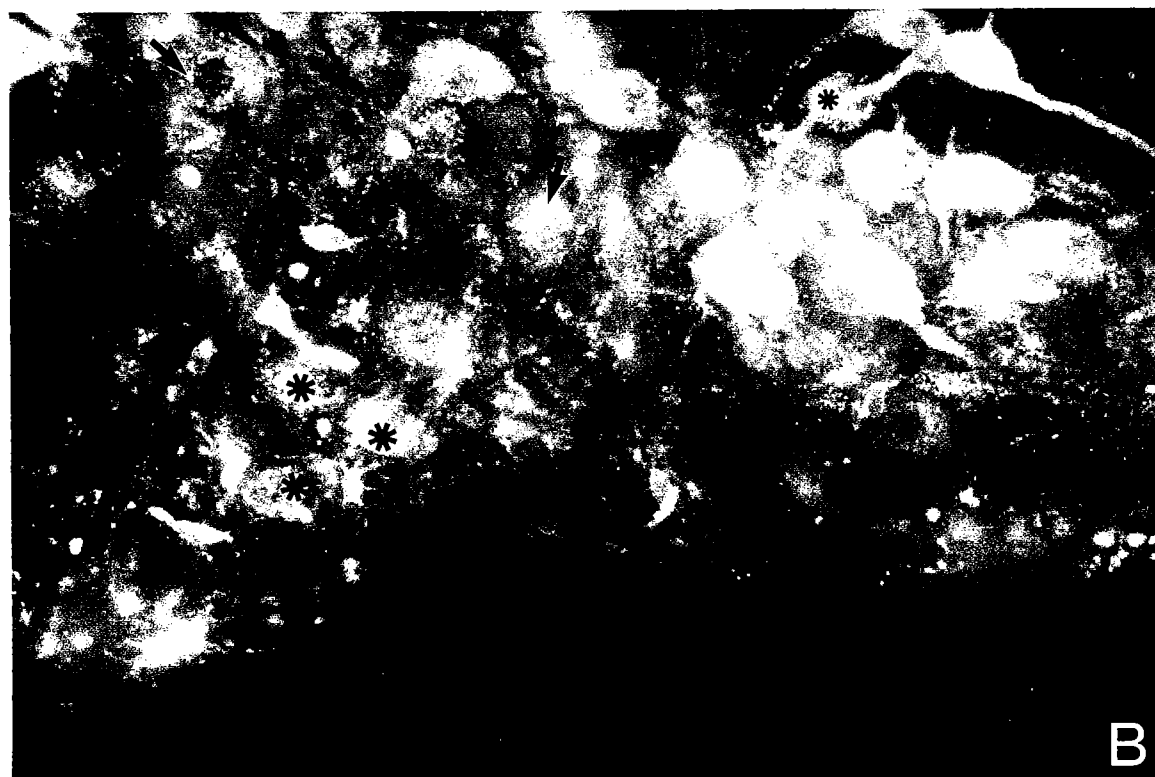
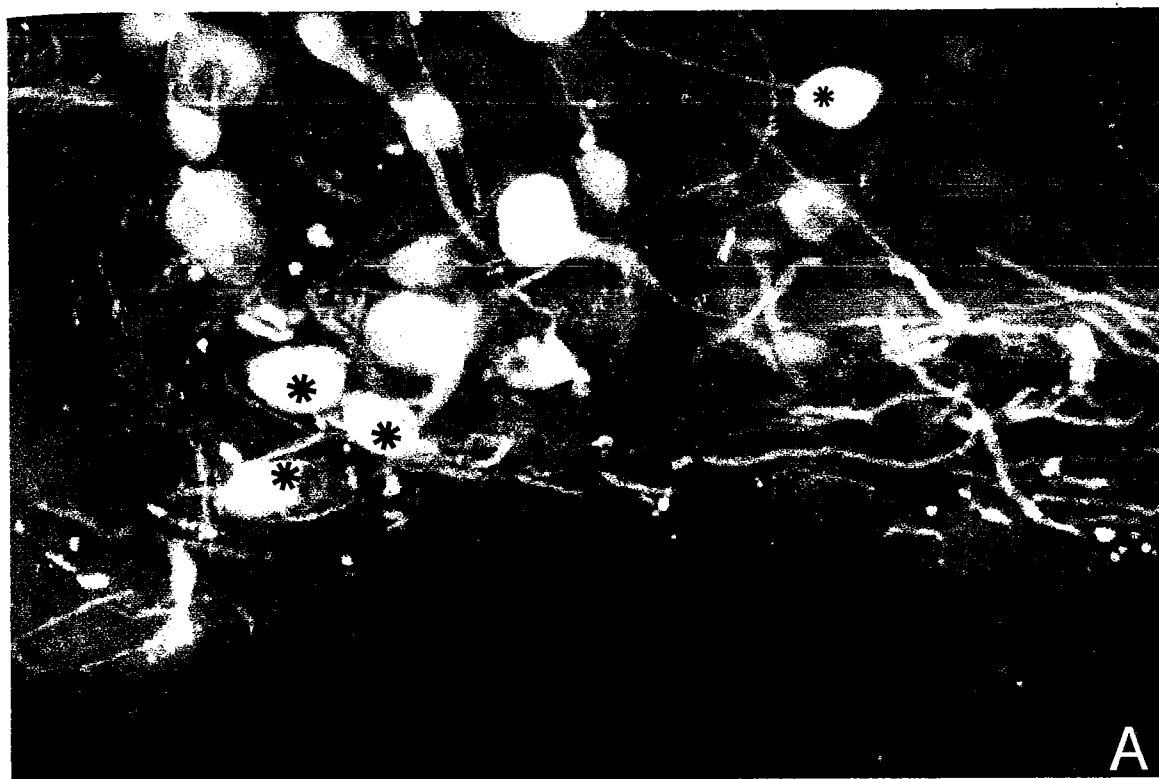


FIGURE 12. Coronal sections (20x) through the middle portion of the PVN for control (A, C), and neurohypox (B, D) animals. Sections were simultaneously labeled for fluorogold and AVP. A and C represent the same brain section photographed using a blue excitation light for AVP and a green excitation light for fluorogold. Cells labeled with fluorogold only indicate non-vasopressinergic cells which also project to the neurohypophysis (see arrows).

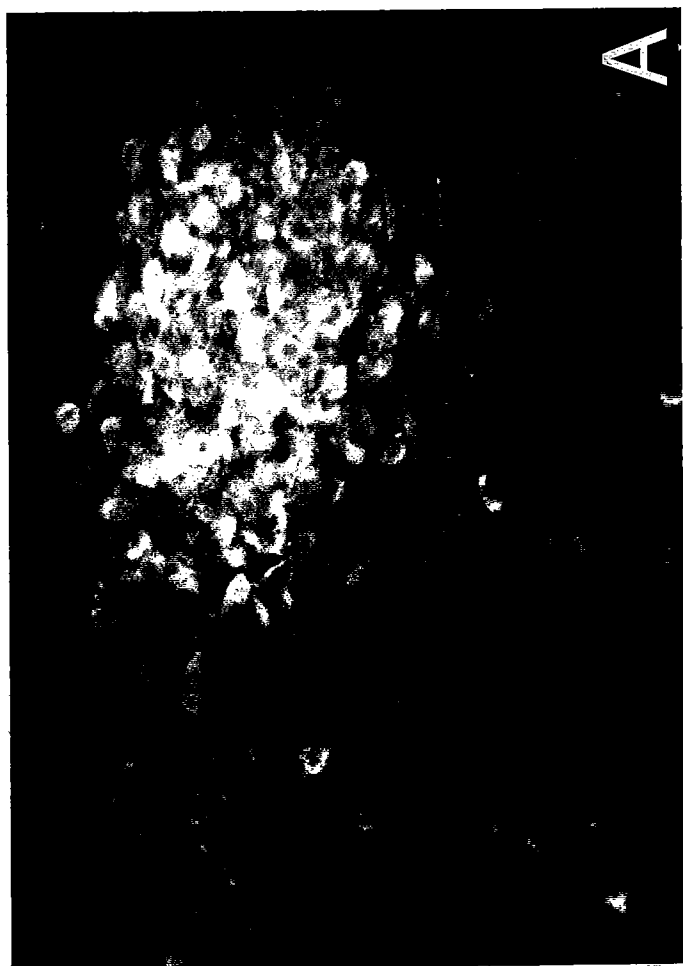
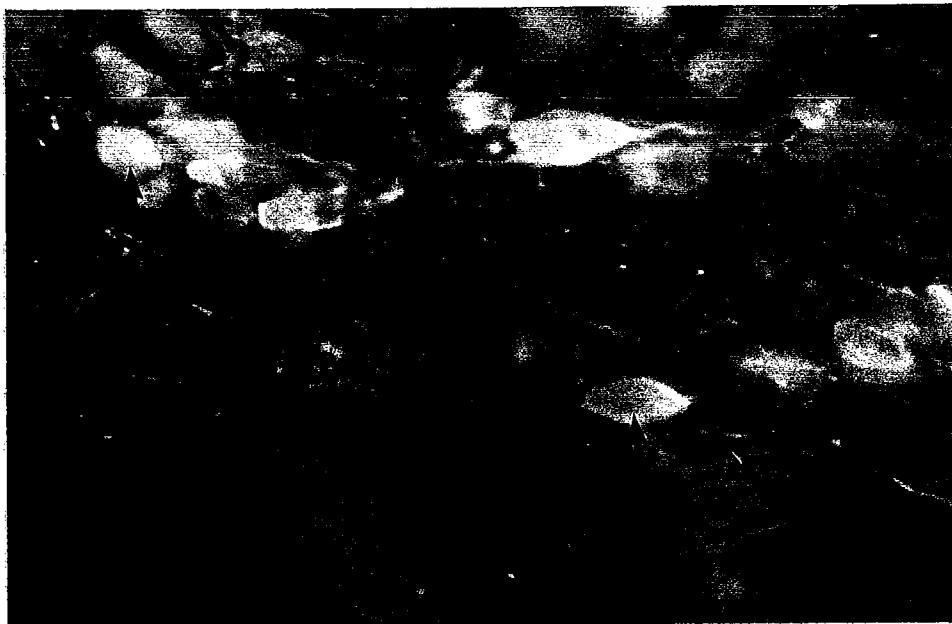


FIGURE 13. Fluorescent micrograph (20x lens) of the ventral area of the posterior magnocellular portion of the PVN. Following neurohypophysectomy (28 days) a small population of cells is co-labeled for AVP and OXY within the same neuron (arrows).

OXY



AVP

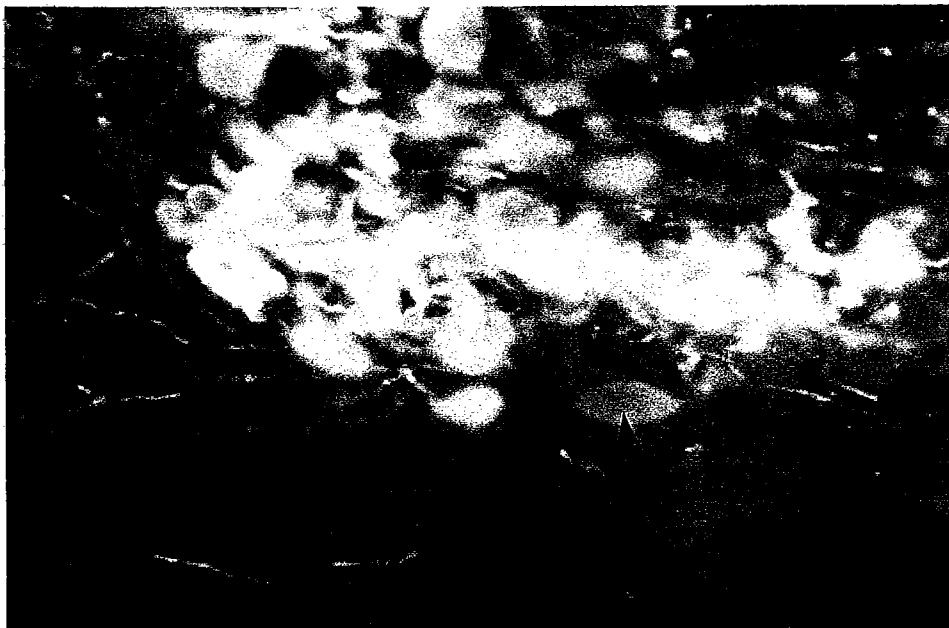
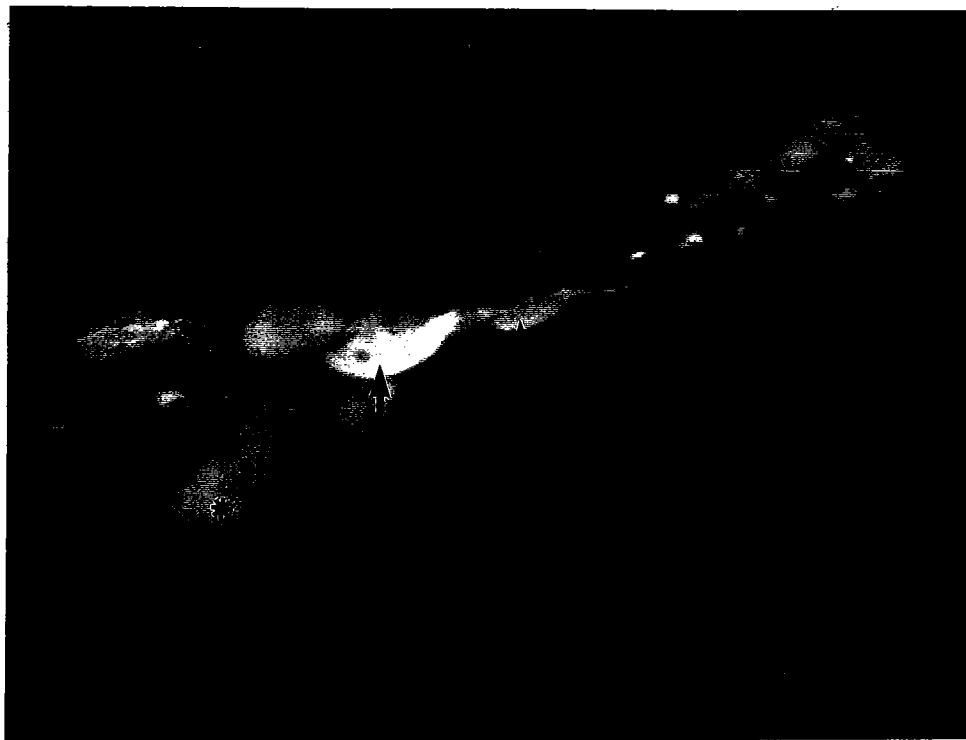
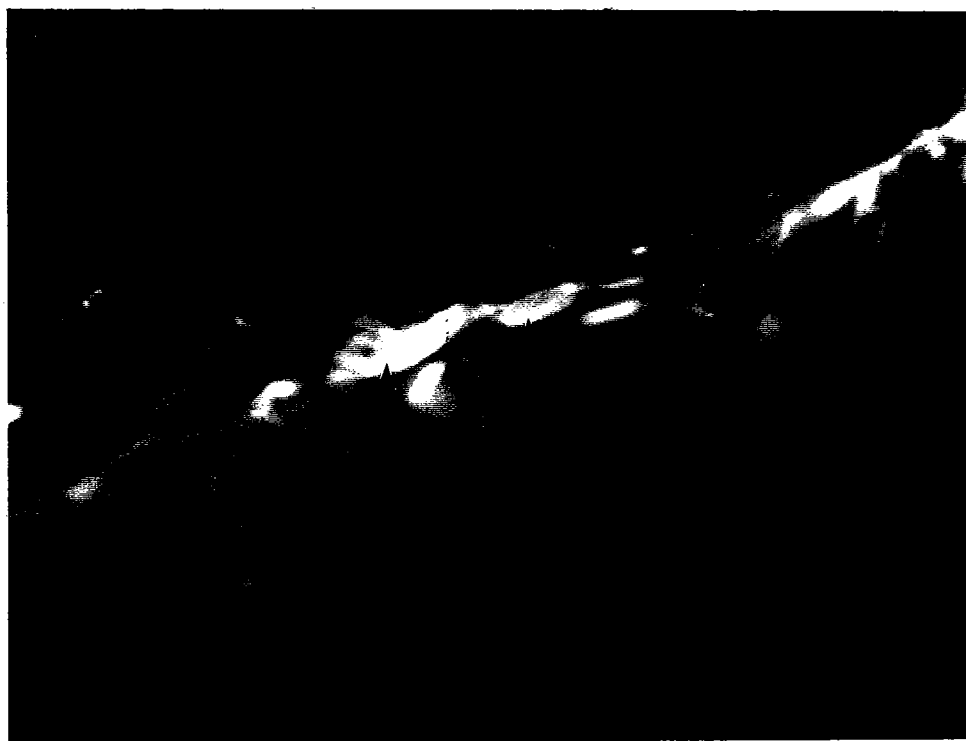


FIGURE 14. Fluorescent micrograph (lens 20x) of the retrochiasmatic area of a neurohypophysectomized rat. Arrows indicate cells which are co-labeled with AVP and OXY. Asterisks are used to suggest more co-localization within cells out of the plane of focus. No co-localization of AVP and OXY was found in normal rats.

OXY



AVP



DISCUSSION

Following neurohypophysectomy there is a moderate loss of AVP immunoreactivity within the magnocellular neurons of the SON versus a dramatic loss of neurons within the SON of hypophysectomized rats. This difference in magnocellular cell loss within the PVN of hypophysectomized versus neurohypophysectomized animals, however, diminishes. The posterior magnocellular area shows a moderate loss of immunoreactivity for both hypophysectomized and neuro-hypophysectomized conditions. Further, retrograde labeling studies with fluorogold, 28 days post neurohypox, support the hypothesis that all magnocellular neurons of the SON project to the neurohypophysis. The preceding studies have shown that there appears to be no compensatory cell populations which synthesize AVP within the SON or PVN in response to neurohypophysectomy. Further analysis within extrahypothalamic areas such as the retrochiasmatic region, however, have provided some initial data suggesting the possibility that cells outside of the HNH system may respond to alterations within the HNH system or to changes in AVP levels within the general circulation. Whether the response is a direct or indirect result of injury within the HNH system remains to be determined.

The topography of the hypothalamo-neurohypophysial system has been examined by many researchers using various labeling methods. The retrogradely transported horseradish peroxidase (HRP) has been used to demonstrate the distribution of neurons projecting to the neurohypophysis from various regions of the brain (Kelly et al., 1980, Armstrong et al., 1980) and in various species (Lupi et al., 1988, Caverson et al., 1987, Ferris et al., 1992). While some studies report a direct injection of HRP into the posterior lobe, many of these studies have injected HRP either peripherally or stereotaxically using Paxinos coordinates (Kelly et al., 1980). Peripheral injections label all neurons non-

selectively. Stereotaxic injections into the neurohypophysis from the dorsal approach are very difficult and may lead to non-specific labeling. Neighboring fibers of somata may be labeled as dye flows up along the needle due to capillary action. Our approach is unique in two ways: 1. the neurohypophysis is directly visualized in order to inject tracer material, and fluorogold, not HRP, is used to retrogradely label those neurons which project into the posterior lobe. And 2. the vasopressinergic system (SON and PVN) is examined only after restoration of the HNH system following neurohypophysectomy.

There are several obvious advantages to visualizing the neurohypophysis before injecting any tracer: the anatomy of the area is such that injections using stereotaxic coordinates results in more misses than not, often injection sites are too large and involve at least part of the intermediate lobe and/or anterior lobe (Price et al., 1978), and in the case of fluorogold, with ultraviolet light you can directly visualize the loading of the neurohypophysis with tracer. Using fluorogold instead of HRP is also more favorable because it is not taken up by fibers of passage, it has a much more intense emission, it will not diffuse or leak out of labeled cells, it is compatible with fluorescence immunohistochemistry allowing for double, possibly triple cell labeling, and most importantly for this study, fluorogold has a long survival time of up to two months, unlike HRP (Schmued, 1986). We injected a total of 2 μ l of fluorogold per animal into the neurohypophysis using two injection sites because axons terminals are selectively oriented within the lobe. SON axons are located more centrally within the lobe and PVN terminals more peripherally (Alonso et al, 1981).

We used only neurohypophysectomized animals for this retrograde tracing study and not hypophysectomized animals because, as discussed in Chapter III, it provides a cleaner model system in which to study the response of the neurosecretory to injury. Following a physiological stress such as the withdrawal of adrenal steroids (i.e.

hypophysectomy), CRF labeled cells have been shown to preferentially enhance the expression of AVP (and CRF) in parvocellular division of neurosecretory neurons within the PVN (Sawchenko et al., 1984). While our results confirm other studies which have also reported that virtually all neurons of SON project to the neural lobe (Armstrong et al., 1980, Raisman, 1973), they don't rule out the possibility that some SON neurons have collateral projections to the lateral hypothalamus that protect them from degeneration following lesion of their projections to the neurohypophysis (Alonso et al., 1996). It would be very interesting to determine if those neurons which survive neurohypophysectomy are indeed the same cells which also have projections to the lateral hypothalamus. Neurohypophysectomy followed by double site injections (into the lateral hypothalamus and the neural lobe) may provide an answer. Also, the presence of some fluorogold-positive/AVP-negative stained cells within the SON may result from the down-regulation of AVP synthesis in response to interruption of the HNH system.. Future studies are necessary to address this possibility.

Fluorescent studies of the PVN using a long-lived retrograde tracer were initiated to study the possibility that parvocellular neurons of the PVN might be capable of expressing or over expressing AVP in response to neurohypophysectomy. Silverman and Zimmerman (1986) showed compensatory sprouting of intact contralateral parvocellular neurons into a denervated region of the external zone of the ME following unilateral lesions of the PVN in adrenalectomized rats. Also, Watts et al. (1995) suggested the existence of several phenotypically distinct sets of neurons within the medial parvocellular division of the PVN, each characterized by its ability to regulate the expression of neuropeptide genes in a stimulus-specific manner. CRF synthesizing cells can, under a variety of circumstances, also synthesize other neuropeptides including AVP (Sawchenko et al., 1987, Whitnall et al., 1989). Based on these findings, as well as the

reported presence of extensive interdigitation of dendrites from magnocellular neurons and parvocellular neurons (Van Den Pol, 1982), we hypothesized that neurohypophysectomy might induce the expression of AVP in some or all of the parvocellular population of neurons.

The major results of this study, in general, support the hypotheses that virtually all magnocellular neurons of the SON project to the neurohypophysis, and that loss of cell numbers in this area is greater in hypophysectomized animals than neurohypophysectomized animals. As anticipated hypophysectomy results in a greater number of AVP stained neurons within the parvocellular area of the PVN.

As previously reported, the SON of hypophysectomized rats was markedly reduced in the number of AVP positive cells, and there was no evidence to support the putative existence of a *de novo* population of cells recruited to express AVP in response to the stress of neurohypophysectomy. The AVP positive magnocellular neurons of the PVN were also affected in the hypophysectomized model but to a lesser degree. In the hypophysectomized model, AVP immunoreactive cells were often observed within the medial area of the PVN along the third ventricle. These correspond to a parvocellular region of the PVN containing CRF cells capable of expressing AVP in response to steroid withdrawal. The neurohypophysectomy model revealed a more controlled loss of AVP expression and suggests the absence of any CRF, parvocellular involvement in the restoration of neurosecretory function.

The putative involvement of other accessory nuclei and non-vasopressinergic neurons of the PVN, i.e. oxytocinergic neurons, expressing AVP in response to axonal injury has been hypothesized. During the mid 1970's immunohistochemical localization of AVP and oxytocin (OXY) in cell bodies of the hypothalamus and in the efferent neurosecretory fibers originating from the nuclei to their terminations in the neural lobe

seemed to support the "one-neuron-one-hormone" concept (Vandesande et al., 1975). Since then several reports have been published which demonstrate the ability of one neuron to synthesize both AVP and OXY hormones under varying conditions (Sokol et al., 1976, Baldino et al., 1988) and reports that neurons may release more than one active substance are expanding for all areas of the CNS.

Following lesions of the septal nuclei and adrenalectomy, the presence of AVP and AVP mRNA were found co-localized with OXY within a few neurons of the medial portion of the posterior PVN and accessory PVN (Baldino et al., 1988). AVP mRNA was detected in a population of neurons that do not normally express AVP or AVP mRNA. This small number of co-localized cells suggest that the CNS possesses the ability to induce peptide synthesis *de novo* in response to specific synaptic events. AVP and OXY co-localization has also been found in normal and heterozygous Brattleboro rats suggesting that some cells of the SON and PVN may have the capacity to synthesize the two hormones (Sokol et al., 1976).

In this study, we used double fluorescent immunohistochemistry to label brain sections from 28 day, post neurohypophysectomized animals. Monoclonal antisera to OXY, and polyclonal antisera to AVP were used simultaneously. Although there was a prevailing lack of double staining for AVP and OXY within both the SON and PVN, we noted a very small population of cells (n=2) which appeared to be co-labeled for both peptides. The decreased intensity of AVP fluorescence within these cells we hypothesize may be due to a lower level of AVP expressed (Fig. 13). A series of control sections were added including the pre-absorption of AVP peptide with the antisera. No labeling could be detected in the suprachiasmatic nucleus suggesting minimal if any cross reactivity with the OXY monoclonal antibody.

In addition to co-localization within the PVN, we also noted a more robust double staining within what is known as the retrochiasmatic nucleus (Fig. 14). Because these immunopositive cells are located very ventral, hence on the edge (in some cases) of the tissue sample, getting all the labeled cells in focus was not possible, therefore we used asterisks to suggest the shadows of other labeled cells. (Again this observation was only made for two animals but with the same controls as mentioned above.)

The retrochiasmatic area, is considered by some to be an extension of the SON (Marciano et al., 1989) and has been somewhat overlooked in regards to the HNH system but it quite interesting when one considers that numerous peptidergic fibers from both hypothalamic nuclei enter the median eminence via this region. It may be the case that these AVP- and OXY-positive cells are simply accumulating peptide from neighboring fibers or they are indeed capable of synthesizing the two peptides simultaneously. Obviously, more animals must be analyzed using immunohistochemical techniques as well as *in situ* hybridization.

While the number of animals analysed must be increased and only a very small number of AVP and OXY positive cells were identified within the retrochiasmatic nucleus with even fewer cells within the ventral, posterior portion of the PVN of hypophysectomized rats, these initial data may support the hypothesis that some non-vasopressinergic cells do have the capacity to synthesize AVP and/or OXY in response to neurohypophysectomy. No similar cells were identified when analyzing intact animals (n=3).

MATERIALS AND METHODS

Young adult male (250-300g) Sprague Dawley rats were used for this study. They were divided into four groups: (1) intact animals (n=3); (2) Hypophysectomized animals (n=3); (3) neurohypophysectomized (n=3); and (4) fluorogold injected, neurohypophysectomized animals (n=7). All animals were fed standard laboratory rat chow and allowed water ad libitum. Following surgery, animals were given 5% sucrose ad libitum and 5 cc s.c. injections of Ringer's solution for 3-5 days.

Surgical Procedures

After deep anesthesia using ketamine/rompum (1cc/100g), the animals were placed in the supine position in a stereotaxic device. Strap muscles were moved aside, a tracheal tube inserted, the ventral surface of the basosphenoid bone exposed and a small hole (2-3 mm in diameter) drilled midline beneath the site of the hypophysis. Once the pituitary gland was exposed, a small incision was made in the dura, the anterior lobes were carefully bisected and the underlying neural lobe gently aspirated out (Ben-Jonathan, 1982). The excised lobe was inspected for its entirety, the drilled site packed with Surgicel, the trachea sutured, and the skin area closed with 9mm wound clips. This procedure caused minor bleeding with minimal damage to the pituitary stalk. In the case of total hypophysectomy, the entire gland was gently aspirated out and again inspected for completeness.

Fluorogold Injections

Before group 4 was neurohypophysectomized, animals received 4% fluorogold dissolved in 0.9% saline 5 to 7 days prior to lobe removal. The neurohypophysis was exposed using the paratracheal approach described above. The fluorogold was injected

into the neurohypophysis using a micrometer driven plunger acting on a 1ml Hamilton syringe connected by a short piece of polythene tubing to a glass micropipette tip. A total of 2 μ l was infused at two different sites within the lobe over a period of 25-35 minutes. Following infusion, the animals' wounds were closed using 9mm wound clips and the animals were kept warm until they recovered from the anesthetic. All animals were allowed standard rat chow and 5% sucrose solution ad libitum while recovering. Once animals had sufficiently recovered (5-7days), the neurohypophysis was removed leaving the anterior lobe intact.

Tissue Fixation and Preparation

After 28 days post surgery, surviving animals were perfused transcardially with 2.5% acrolein (EM Grade, Polysciences, Warrington, PA) and 4% paraformaldehyde in 0.1M potassium phosphate-buffered saline (KPBS) (Berghorn et al, 1994). After fixation, the brains were removed and placed into 25% sucrose in KPBS at 4 °C until they sank. The brains were then cut on a freezing microtome, 35-45mm, and stored in cryoprotectant solution (Watson et al., 1986.) at -20 °C until they were processed for immunostaining.

Immunohistochemical Procedures

Conventional immunoperoxidase staining was used to label brain sections for camera lucida drawings. Briefly, free floating brain sections were repeatedly rinsed in KPBS to remove cryoprotectant. Next, sections were incubated with 1% sodium borohydrite (10 minutes), rinsed repeatedly in KPBS and preincubated in 20% normal goat serum, 0.3% triton-X 100, 1% hydrogen peroxide in Tris-buffered saline (TBS), pH 7.6, at room temperature (RT) for 30 min. Next, tissue was incubated in diluted

(1:16,000) primary AVP antisera (ICN, Costa Mesa, CA). Following the primary incubation and a series of washes in KPBS, a Vectastain ABC Elite kit (Burlingame, CA) with diaminobenzidine (DAB) (Sigma, MO) was used to detect any reaction product. Sections were thoroughly rinsed, mounted on gelatin coated slides, dehydrated in a series of alcohols, and cover-slipped using Permount (Fisher Scientific).

Fluorescent Immunohistochemistry

This procedure is quite similar to conventional immunoperoxidase staining. Briefly, free floating, acrolein-paraformaldehyde fixed sections were processed as described above and were incubated with primary antisera to fluorogold (1:500, Chemicon Int'l, CA) and monoclonal anti-AVP (1:400, CCA-223, the generous gift of Dr. A.J. Burlet) simultaneously. Sections were incubated for approximately 48 hours at 4 °C on a gentle shaker. Next, sections were rinsed well then fluorescently conjugated immunoglobins; 25ml rabbit IgG- Texas Red-X (Molecular Bio Probes, OR) and 25ml mouse IgG-Fluorescein Isothiocyanate (FITC) in 5ml KPBS were added. Sections were incubated in the secondary antibody wash for 1 hour at room temperature. Once repeatedly rinsed, the sections were then mounted on gelatin coated glass slides and cover slipped with Krystalon. Double staining for AVP and OXY were carried out as described above. The monoclonal antisera for OXY (A1-28) was the generous gift of Dr. H. Gainer. Polyclonal antisera for AVP (1:16,000) and monoclonal antisera for OXY (1:200) were used simultaneously in a 4 °C overnight incubation.

Camera Lucida

Atlases were drawn directly from AVP-stained, 35mm, coronal sections with the aid of a Zeiss light microscope (10x lens). The atlases for both the SON and PVN (figs. 7 and 8) were representative of at least three brains for each group. Sections were drawn by an independent observer then verified by the authors.

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CHAPTER IV

LOCALIZATION OF VASOPRESSIN IMMUNOREACTIVITY IN GLIA OF THE THIRD VENTRICLE OF NEUROHYPOPHYSECTOMIZED RATS FOLLOWING OSMOTIC STIMULATION

INTRODUCTION

One of the major neuropeptides expressed within the hypothalamus by the magnocellular neurons of the SON and PVN is AVP (Zimmerman et al., 1974). Several reports show that AVP immunoreactivity and its mRNA are found in extrahypothalamic areas following neurohypophysial tract transection and in response to osmotic stress (Mohr et al., 1990, Murphy et al., 1989, Richter et al., 1985, Pu et al., 1995). In particular, Pu et al. (1995) reported the ability of pituicytes, a modified subtype of astrocyte, to synthesize AVP mRNA within the neural lobe following stalk transection and osmotic stimulation (Pu et al., 1995, Boersma et al., 1993). Although these cells do not provide the major source of AVP mRNA, they are able to translate and up-regulate AVP mRNA in response to osmotic stimulation. Astrocytes, in vivo as well as in culture, have also been shown to express other neuropeptides such as proenkephalin (Hauser et al., 1990, Shinoda et al., 1989), angiotensin mRNA (Stornetta et al., 1988), atrial natriuretic factor (McKenzie, 1992), and prosomatostatin mRNA (Shinoda et al., 1992) in response to various stimuli.

These findings led us to ask whether the stress of neurohypophysectomy associated with the loss of AVP and disruption in water homeostasis would facilitate AVP gene expression in astrocytes related to the neurosecretory system. Following a

series of immunohistochemical studies, an interesting pattern of AVP labeling which appeared to encircle the caudal portion of the third ventricle was observed in animals which had been neurohypophysectomized but was absent in normal animals.

Immunohistochemical procedures using antisera to glial fibrillary acidic protein (GFAP) and AVP concurrently failed to detect any immunoreactivity within the SON or PVN but did demonstrate co-localization of these antigens within astrocytic appearing cells in the wall of the third ventricle.

The presence of AVP immunoreactivity within the periventricular glial cells of the third ventricle may indicate the ability of these cells to respond to osmotic stimulation by up-regulating AVP expression. However, the possibility remains that the AVP immunoreactivity in these cells may be the result of endocytosis from the circulating cerebrospinal fluid, or from diffusion of AVP from neighboring vasopressinergic fibers. To demonstrate AVP mRNA synthesis within these AVP immunopositive cells, we used *in situ* hybridization assays. We also immunostained brain sections using anti-neurophysin-AVP (PS41) in addition to the *in situ* hybridization analysis. The antibody PS41 has been shown to be specific for the carrier protein neurophysin II (Yakov et. et., 1985), hence the positive immunostaining within these cells would support the hypothesis that AVP is indeed synthesized by these periventricular glial cells.

RESULTS

Immunofluorescent histochemistry

Following neurohypophysectomy and a moderate stimulus of 2% saline for 14 days, a small population of AVP positive cells were identified encircling the caudal portion of the third ventricle (Fig. 15A). These cells appeared stellate in shape and virtually all AVP labeling was blocked following a pre-absorption procedure of the antisera with AVP peptide prior to immunohistochemical staining (Fig. 15B).

When anti-AVP and anti GFAP were used simultaneously to label neurohypophysectomized brain sections, no immunoreactivity was found within the SON or PVN but a small population of GFAP-positive cells co-localized with vasopressin-like immunoreactivity within the wall of the third ventricle. Based on their stellate morphology at the light microscopic level and the positive labeling for GFAP these cells were identified as astrocytes (Fig. 16). Although the number of GFAP positive cells co-expressing AVP was small, they were consistently found lying within the ventricular wall just medial to the ependymal layer. The monoclonal antiserum for glial fibrillary acidic protein (GFAP) has been extensively characterized (Debus et al., 1983 and Franke et al., 1991) and has been demonstrated to have minimal cross reactivity with other intermediate filaments proteins.

In Situ Hybridization

The localization of AVP mRNA to ventricular glia using in situ hybridization analysis was inconclusive. While there were some instances of silver grains overlaying parts of the third ventricle, no significant or consistent clustering could be detected (data not shown). As suggested by Pu et al. (1995) the relatively low sensitivity of the technique as well as the relatively small amount of AVP being synthesized within these

glial cells as compared to neurons transporting AVP to the hypothalamus may account for the lack of observable labeling. Thus, detecting a change in expression of such a small population of glial cells would be difficult if basal levels of gene expression are low or even absent. No AVP could be detected in GFAP labeled cells in control animals which had a normal salt load (n=3).

Further immunohistochemical analysis using PS41 was performed to demonstrate the synthesis of AVP within these glial cells. After 14 days of salt loading (2% saline ad libitum) neurohypophysectomized animals, no immunopositive cells were observed within the wall of the third ventricle. Rather, a marked pattern of positive staining was observed encircling the entire posterior portion of the third ventricle (Fig. 17). These ependymal-looking NP-AVP immunopositive cells were further analysed using the mouse monoclonal antibody for nestin (Rat 401). Brain sections processed using this marker for neuronal precursor cells produced a similar staining pattern as that seen for PS41 (Fig. 18). The implications of these findings will be discussed subsequently.

FIGURE 15. Immunofluorescent light micrograph (40x lens) of the caudal portion of the third ventricle of a neurohypophysectomized rat. Panel A shows immunopositive cells for AVP lining the wall of the ventricle. In panel B no labeling could be detected following a preincubation of antiserum with AVP peptide. No AVP-like immunoreactivity was observed within intact animals.

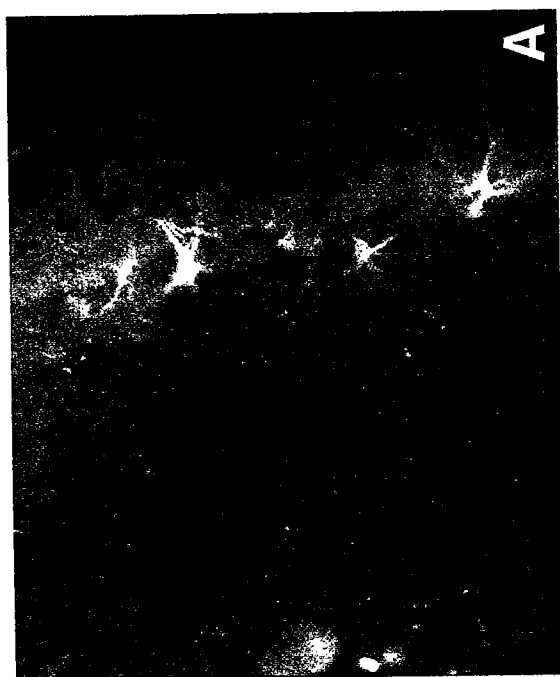
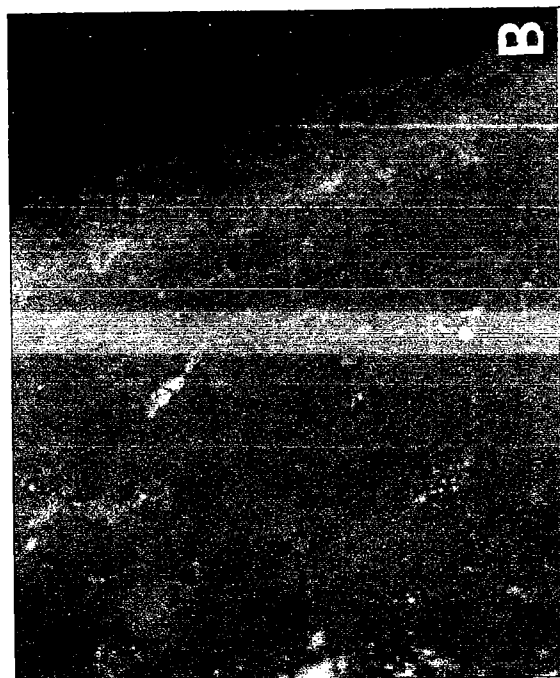


FIGURE 16. Simultaneous detection of AVP and GFAP (100x lens). Panel A shows GFAP immunoreactive glial cells lining the third ventricle of a neurohypophysectomized rat. Panel B shows identical cells immunoreactive for AVP. The arrows in each panel indicate co-labeled cells.

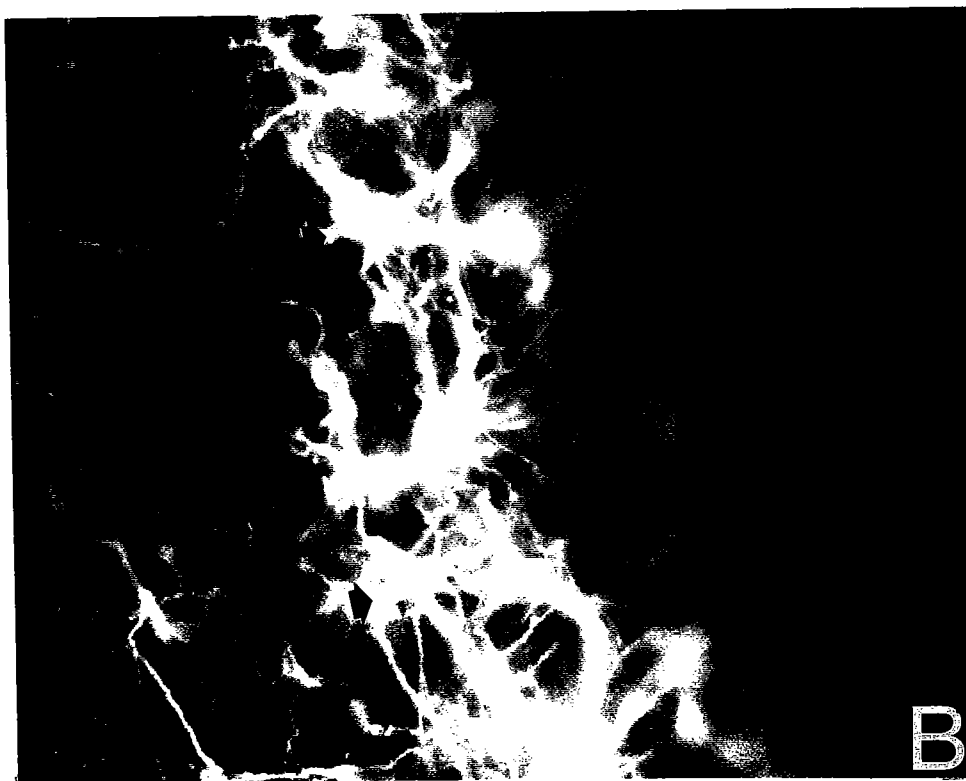
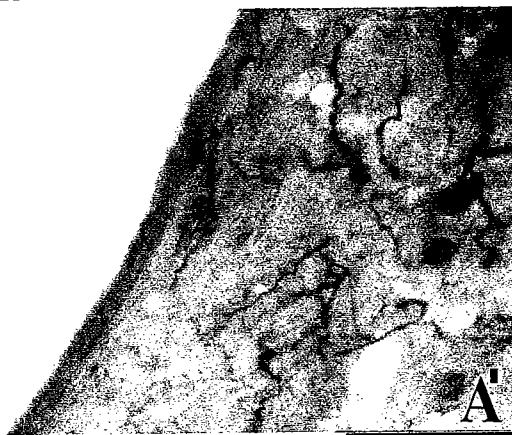
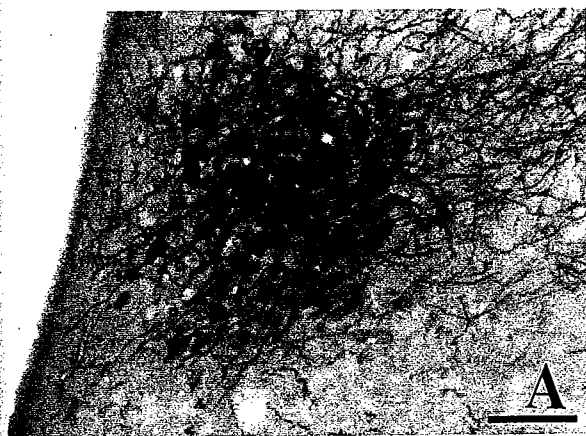


FIGURE 17. Light micrograph, single detection, of anti-neurophysin-AVP (PS41) in a control animal versus neurohypophysectomized (Neurohypox), salt loaded animal.

Immunoreactivity was observed within the caudal portion of the third ventricle. Panels A and B (10x lens magnification) show positively labeled neurons within comparable areas of the PVN. The Panels A' (Control) and B' (Neurohypox) detail the ventricular area (20x). Note the intense immunoreactivity around the ventricle of the neurohypophysectomized animal (arrows). Micrographs represent three animals for each group.

Control



Neurohypox

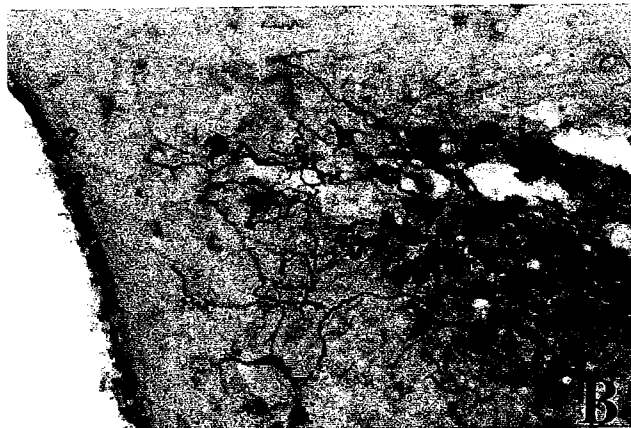


FIGURE 18. Light micrograph (10x lens) of anti-Nestin (Rat.401) immunoreactivity in a normal rat (A) versus neurohypophysectomized, salt loaded animal (B).

Ventricular ependymal cells in control animals (panel A) showed no positive labeling. The ependymal cells lining the ventricle in panel B (neurohypophysectomized), however, are robustly labeled for nestin. (Note: these data represent two animals for each group. Appropriate control tests were performed.) Inset 20x lens

B

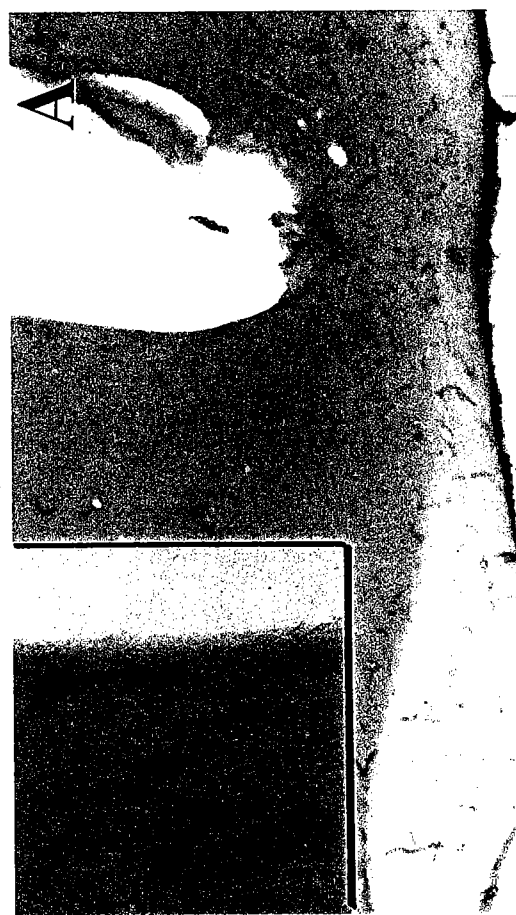
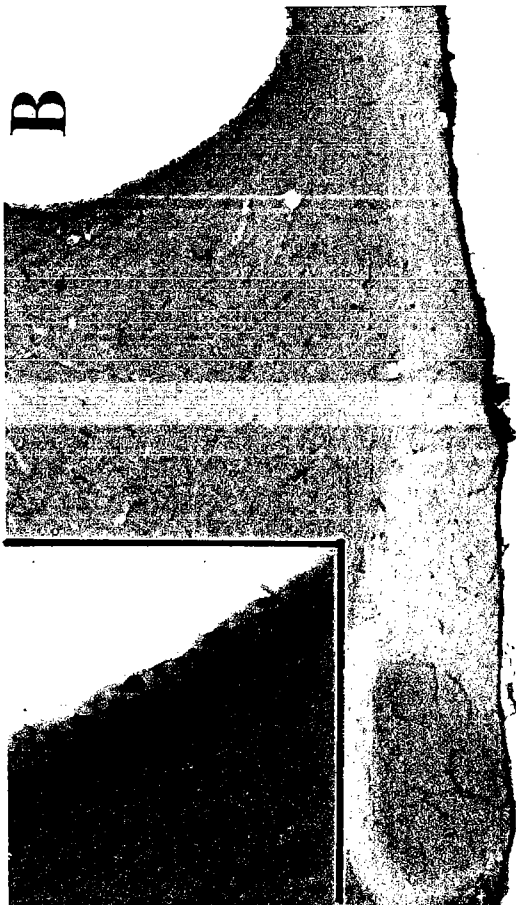
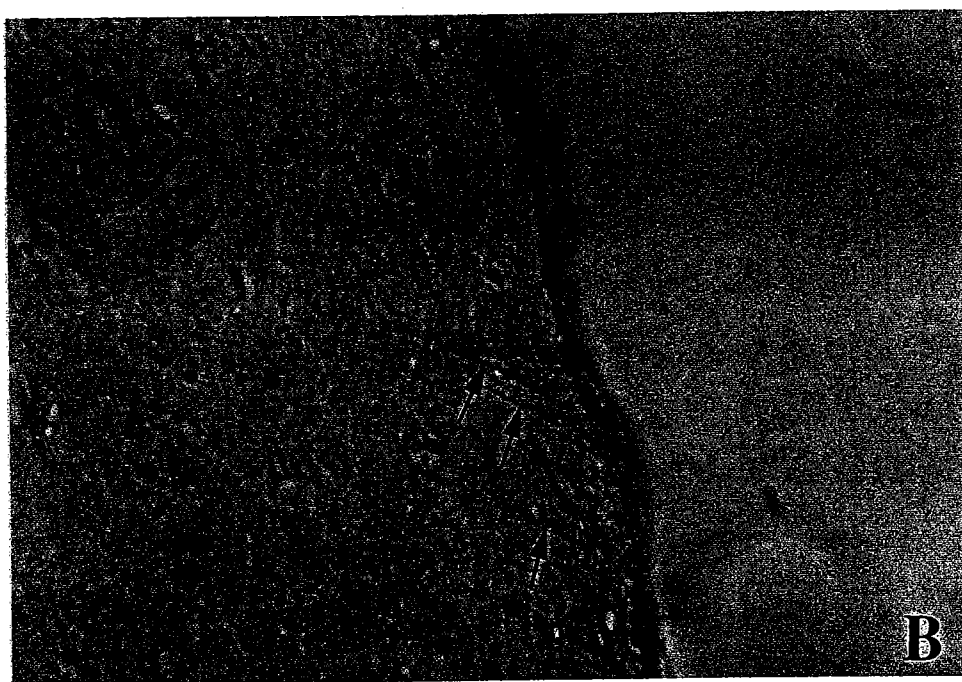
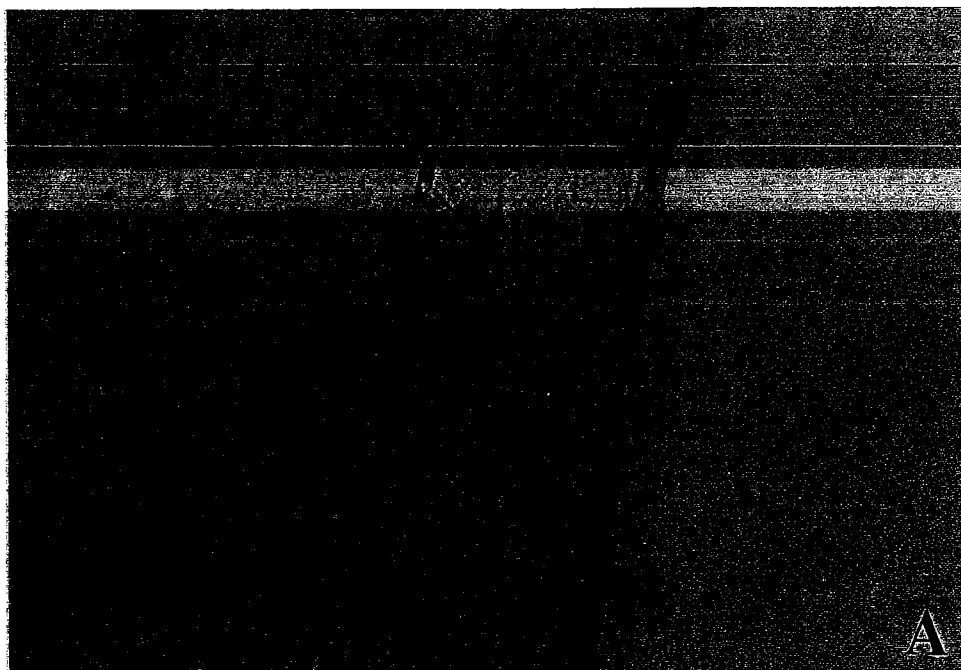


FIGURE 19. Light micrograph of anti-Nestin (Rat.401) immunoreactivity in a neurohypophysectomized rat (B). Using phase contrast microscopy, immuno-positive cells for Nestin (B) also contain thin, Nestin positive fibers (arrows) which seem to project away from the ventricular wall. No fibers were observed projecting from the ventricular cells of normal animals (A). 63x lens magnification



DISCUSSION

In this study, we report the presence of AVP immunoreactivity within periventricular astrocytes of the third ventricle. This immunoreactivity may be the result of endocytosis from the circulating cerebrospinal fluid (CSF) or from diffusion of AVP from neighboring nerve fibers of the PVN (Choudhury et al., 1990). The possibility that these glial cells may in fact be synthesizing AVP as a result of axonal injury and/or osmotic changes in the CSF is most compelling. In light of the fact that our *in situ* hybridization studies were inconclusive, we chose to use AVP-associated neurophysin antisera (PS41) and nestin monoclonal antisera (Rat 401). Using an antibody for the protein carrier which is cleaved from the same hormonal precursor as AVP itself was a logical approach to assessing the possibility that AVP is indeed synthesized and not endocytosed by these glia. Immunohistochemical staining of neurohypophysectomized brain sections using anti-AVP-neurophysin (PS41) however, revealed some interesting results. The neurophysins represent a family of highly homologous, disulfide-rich proteins. Specific neurophysins are associated with either AVP (NP-AVP) or OXY (NP-OXY) in equimolar amounts in each neurosecretory vesicle (Ben-Barak et al., 1985). Vasopressin and NP-AVP are synthesized as part of a common precursor (prohormone) (Brownstein et al., 1980) and are co-released with AVP or OXY from nerve terminals in the neurohypophysis (Seif et al., 1978). Because of this close association between neurophysin and AVP it may be inferred that the presence of NP-AVP (PS41) within a cell is indicative of prohormone synthesis.

Following neurohypophysectomy and salt loading for 14 days, a robust pattern of cell staining for NP-AVP was observed encircling the entire third ventricle (Fig. 17). Interestingly, these positively staining cells did not exhibit the same sparse pattern or

astrocytic appearance as first seen with the GFAP and AVP co-staining. The non-stellate, columnar morphology of these cells and their close association with the ventricular surface suggests the labeling of another type of glial cell: ependyma. Ependymal cells, like astrocytes and pituicytes, are derived from the same neuroepithelium within the developing ventricular zone of the CNS.

In addition to staining neurohypophysectomized brain sections for NP-AVP, we also used the mouse monoclonal Rat 401 antibody to stain for the presence of Nestin. By using this antibody we were able to explore the possibility that ependymal cells of the third ventricle are capable of de-differentiating in response to neurohypophysectomy and expressing early neuronal genes. Antisera to Rat 401 (nestin) recognizes neuronal precursor cells within the central nervous system as well as Schwann cells and other glial cells in the peripheral nervous system (Friedman et al., 1990, Hockfield et al., 1985). Anti-nestin binds transiently to a very large portion of cells in the embryonic brain, such as the mitotic regions of the neural tube. It is localized to proliferating CNS stem cells during embryogenesis (Hockfield et al., 1985). Immediate precursors to neurons also stain positively for Nestin (Frederiksen et al., 1988). The loss of Nestin expression within the developing brain coincides with the terminal differentiation of these early multipotential cells. Neither neurons nor glia in the adult rat brain express the epitope recognized by the Rat 401 antibody (Frederiksen et al., 1988).

In figure 18B are shown ependymal cells lining the wall of the third ventricle intensely labeled following neurohypophysectomy and osmotic stimulation, with no apparent labeling in the intact animal (Fig. 18A). The labeling appears to be quite similar in morphology to that seen for NP-AVP labeling. While these data must be viewed cautiously, and the implications of these findings have yet to be determined, initial results

have yielded a potentially interesting avenue of research and are presented as conjecture for future study.

The Rat 401 antibody has been shown not to cross react with vimentin or GFAP. Recall that anti-vimentin antibodies can recognize ependymal cells, and anti-GFAP antibodies can recognize glial cells. Hockfield and McKay (1985) reported that Rat 401 positive cells had several morphological features similar to radial glia. In figure 19B one may observe small, thin fibers extending from these Nestin positive cells which may represent a radial glial component to these ependymal cells. These fibers seem to be oriented perpendicular to the ventricular surface and look quite similar to radial glial cells of the developing CNS. As previously suggested, these cells may be responding to some unknown factor released as a result of neurohypophysectomy, or osmotic stress, and may be re-expressing some developmentally, down-regulated neuronal genes, e.g. Nestin.

While these NP-AVP and Nestin data do not directly support the hypothesis that AVP is synthesized within astrocytic cells of the third ventricle, the in situ hybridization studies may still yield positive results when implemented with immunohistochemistry simultaneously. The initial immunohistochemical observations of NP-AVP and Nestin also do not directly support the presence of AVP synthesis within the ventricular population of astrocytes, and the pattern of positive staining for both antibodies provide an opportunity for some interesting speculation regarding the adaptability or plastic nature of ventricular cells such as astrocytes and ependyma.

These preliminary results allow one to hypothesize that ependymal cells in a young adult rat may be able to alter their gene expression, (possibly differentiate into another ontogenetically related glial cell) as a result of osmotic changes within the organism. This hypothesis may be supported from work done by Pavel (1975) in which

fetal, neurohypophysial cells, identified as ependymal cells, released an active antidiuretic substance into the culture medium.

Finally, the presence of NP-AVP and the absence of AVP immunoreactivity within the ependymal cells, as well as the reverse within the astrocytic cells of the third ventricle, are intriguing. However, further studies must be conducted to rule out other sources of non-specific labeling or cross reactivity before any conclusions may be drawn. Antisera may not recognize the peptides if they contained amidated forms or were processed into smaller forms. The initial results reported presently may indeed reflect a real difference in the processing of AVP precursor-derived peptides in ependymal cells or astrocytes.

We believe it unlikely that the immunoreactivity in these astrocytes is the result of diffusion from nerve terminals or damage due to histological preparation. Positive staining for vasopressin was only present in a small number of the many astrocytes found within the area. Moreover, there exists mounting evidence to implicate neuropeptides as likely candidates for neuronal-glial signaling in hypothalamic centers which may undergo remodeling (Montagnese et al., 1990, Theodosis et al., 1993). Neuronal-glial signaling in extrahypothalamic cell groups, i.e. the periventricular cells surrounding the third ventricle, may also influence *de novo* gene expression within these cells.

Various reports have suggested that astrocytes play a dynamic role in the control of hormone synthesis and its release from the central nervous system (CNS) (Hatton et al. 1984). Under certain conditions, such as in response to activation or mitogenic stimuli, astrocytes have been shown to express several different neuropeptides (Boersma et al., 1993). While the mechanisms by which this occurs have yet to be elucidated, it is possible that astrocytes which line the third ventricle and are exposed to the CSF may be able to respond to osmotic changes in the CSF. Pituicytes, i.e. astrocytes of the

neurosecretory system, have also been shown to react to axonal injury by retracting their perivascular end-feet (Theodosios et al., 1986, Raisman, 1973). This presumably allows axonal endings access not only to each other but to perivascular space, leading ultimately to increased hormone release (Raisman, 1973, Tweedle et al., 1977). It is also possible that these astrocytes are influenced by neurosecretory cells of the lateral PVN.

Choudhury (1990) has described the presence of an elaborate vasopressinergic network which interconnects the ependymal, periependymal, and PVN areas. Such a network, in addition to osmotic changes within the CSF, may provide an indirect route by which glial cells of the third ventricle are signaled by injured axons of the PVN to express AVP in response to neurohypophysial damage.

In conclusion, the data presented here suggest the presence of extrahypothalamic cells which are capable of synthesizing AVP in response to neurohypophysectomy and/or salt loading. Whether the AVP is released into the CSF and whether the amount of AVP produced by these cells has any effect on the neurohypophysectomized rat are questions not within the scope of this work.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 175 - 200g and given access to food and water ad libitum were used in these studies. For salt loading experiments, animals were given 2% saline ad libitum for two weeks following neurohypophysectomy. Animals were allowed a surgical recovery period of three to four days before starting saline treatments. Animals were neurohypophysectomized in our laboratory using a paratracheal approach. Briefly, animals were deeply anesthetized and placed in a stereotaxic apparatus in the supine position. Strap muscles were moved aside, a tracheal tube inserted, the ventral surface of the basosphenoid bone exposed and a small hole (2-3mm in diameter) drilled midline beneath the site of the hypophysis. Once the pituitary gland was exposed, a small incision was made in the dura, the anterior lobes were carefully bisected and the underlying neural lobe gently aspirated out. The excised lobe was inspected for its entirety, the drilled site packed with surgicel, the trachea sutured, and the skin area closed with 9mm wound clips (Ben-Jonathan, 1982). This procedure caused minimal bleeding and did not damage the anterior lobe or the pituitary stalk.

Immunohistochemistry

Rats were deeply anesthetized with ketamine-rompun (0.3 cc/100 g) and transcardially perfused with 5,000 units heparin (1cc), followed by 4% paraformaldehyde in 0.1M phosphate buffer pH 7.4. Brains were post-fixed for 1-2 hours then placed in 25% sucrose overnight. Using a freezing microtome, brains were cut in the coronal plane at 30 μ m. Sections were incubated with anti-AVP (ICN, Costa Mesa, CA) and anti-

GFAP (Sigma Laboratories, St. Louis, MO) simultaneously. Briefly, sections were pre-incubated in normal goat serum (NGS), 0.3% triton-X 100, 1% hydrogen peroxide, and Tris-buffered saline (TBS) pH 7.6 at room temperature for 45 minutes. Next, tissue was placed in primary antisera: AVP, (1:2000) and GFAP, (1:400) for 48 hours at 4 °C, on a gentle shaker. Following several washes in buffer (2% NGS, 3% triton-X 100, 95% TBS), fluorescently conjugated secondary antibodies were then added: goat anti-mouse immunoglobulin conjugated to fluorescein isothiocyanate (FITC) and goat anti-rabbit immunoglobulin conjugated to Texas Red[®]: 25 µl in 10 ml wash buffer gently shaken for 1 hour at room temperature. After rinsing well, sections were mounted on gelatin coated slides, and cover-slipped using Krystalon mounting media.

In addition to omitting primary antibodies for each peptide respectively, a preabsorption control for AVP was also performed (Fig.15B). Approximately 0.25 mM of AVP peptide was incubated with free floating brain sections at 4 °C overnight. Immunohistochemical procedures continued as stated previously.

Fluorescent Microscopy

Fluorescently labeled tissue sections were analyzed separately with a Zeiss microscope under blue and green excitation light for FITC and Texas Red respectively. A 100x, oil-immersion lens and Zeiss 100 spot camera with 400ASA color film (Kodak Elite) were used. Exposure times varied between 15-50 seconds.

In Situ Hybridization

Brains were rapidly removed from deeply anesthetized rats and placed in chilled isopentane and stored at -80 °C until use. Coronal brain sections were cut at 20mm on a cryostat, thaw mounted on RNase-free gelatin subbed slides, and stored at -80 °C.

Hybridization was conducted as previously described (Melloni et al., 1994). Briefly, slides were warmed to room temperature and post fixed in 4% paraformaldehyde (pH 7.4), then rinsed in PBS (pH 7.4), washed in acetic anhydride (25% in 0.1M triethanolamine), then dehydrated and delipidated through a graded series of alcohols and chloroform. The AVP probe, a single stranded 48-base oligonucleotide sequence complementary to the region of the AVP mRNA encoding the last 16 amino acids, was 3' end labeled with [35S] dATP (New England Nuclear) using terminal deoxynucleotidyl transferase. It was purified by ethanol precipitation, and then heat denatured. The probe was diluted in hybridization buffer containing 50% formamide, 10% dextran sulfate, 0.3M NaCl, 10mM Tris (pH8.0), 1mM EDTA, 1x Denhardt's, 0.5mg/ml yeast tRNA, and 10mM dithiothreitol. The probe was applied to the tissue at a concentration of 2.5pmol/ml, previously determined to saturate mRNA in the PVN and SON (Ferris et al., 1995). Slides were covered and placed in a moist chamber over night at 37 °C.

Following the over night incubation, slides were washed four times in 1x standard saline citrate (SSC) at 55 °C, followed by two washes in 1X SSC at room temperature for one hour each. The tissue was rehydrated through alcohols containing 0.3M ammonium acetate and then dipped into Kodak NTB2 nuclear track emulsion (diluted 1:1 with 0.6M ammonium acetate). Once air dried completely, the slides were stored at 4 °C for 7 to 30 days. They were then brought to room temperature and developed with Kodak D-19 (Eastman-Kodak, Rochester, NY), stained with cresyl violet, and cover slipped for analysis.

Neurophysin/Nestin Immunohistochemistry

Anti-neurophysin II (PS41) has been extensively characterized (Yakov et al., 1985) and all pertinent controls were performed. Immunohistochemistry using the

monoclonal antibody for neurophysin II, (PS41) (1:250) was carried out as described in Chapter 3 with some slight modifications. Instead of fluorescently labeled secondary antibodies, the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) was used in combination with diaminobenzidine (DAB) for antigen detection . Following the final rinse, brain sections were mounted on gelatin coated slides and cover slipped with permount. Neurohypophysectomized brain sections labeled for the presence of nestin were also carried out as described in Chapter 3. Following the preincubation period with normal goat serum, partially purified anti-nestin (1:1000) (Developmental Studies Hybridoma, Iowa City, IA) was added. After 2 days gently rocking at 4 °C and a series of washes, the secondary-biotinylated goat anti-mouse antibody was added. Antigen was detected using the Vectastain Elite ABC kit and DAB.

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CHAPTER V

DISCUSSION / SUMMARY

Numerous investigators have reported on the ability of the hypothalamo-neurohypophysial system to regenerate following injury to axon terminals (for a review see Delman, 1973). Following hypophysectomy, accumulation of neurosecretory material at the proximal ends of severed axons, enlargement of the distal portion of the median eminence, angiogenesis of vasculature from the external zone of the median eminence, and cellular re-organization within the SON and PVN, have all been described repeatedly. However, the time course involved in these events and the extent of cell loss in both the PVN and SON, have varied widely. Here we have reassessed several parameters of the regenerating neurosecretory system. We define the partial recovery of the system as the re-appearance of AVP immunoreactivity within the median eminence, concomitant with measurable levels of plasma AVP and decreased water intake/output. This partial recovery occurred no sooner than 28 days post-hypophysectomy. The extent of cell loss within the neurosecretory system as determined by immunohistochemistry and detailed cellular maps has also further defined the system by ruling out the possibility that other *de novo* cells in the SON and PVN may contribute to the release of AVP following lobe removal. This reassessment of the time required for partial restoration is important to establish before identifying any peptides, neuropeptides, or trophic factors which may or may not correlate with the regrowth of vasopressinergic axons and functional release of AVP into the circulation following lobectomy. For example, Alonso et al. (1995) reported that growth associated protein (GAP-43) is not essential for the post lesional axonal growth and regeneration of vasopressinergic axons. It is possible that

their results are misleading. They observed GAP-43 immunoreactivity on days 5, 10, 15, and 30 post lesion. In our studies, the important period of peptide expression occurred between days 21 and 28 post axotomy. Immunoreactivity for AVP and synapsin I was virtually absent within the median eminence of experimental animals on day 21 post-hypophysectomy then markedly noticeable when examined one week later. This "window of reorganization" may have been overlooked in the case of GAP-43. A later time point would be necessary for a final evaluation of GAP-43's role in vasopressinergic axon re-growth.

In addition to describing when the HNH system is partially restored, those cells which contribute to the restoration of vasopressin function following neurohypophysectomy were identified (Chapter 3). Many studies have described the injured system using retrograde tracers within the first few days of cellular response but few if any have waited to examine the remaining cell populations after some degree of water balance had been established. Identifying the cell populations which project to the neurohypophysis exclusively and others which may express AVP but not project to the neurohypophysis is important before one can equate the expression of neuropeptides, neurotransmitters or other markers of plasticity with axonal regenerative processes within the hypothalamus following disruption of the neurosecretory system. Our approach has enabled us to investigate the role of other neurons putatively involved in the functional restoration of the HNH system.

The regenerative responses of magnocellular neurons following injury have been shown in a variety of conditions (See Watt and Paden, 1991, for review). Watt and Paden (1991) have indicated that uninjured vasopressinergic cells are able to respond to axotomy. Continuing with this hypothesis, we investigated the possibility that other uninjured, hypothalamic cells may also be able to respond to axotomy by synthesizing

AVP or in the case of parvocellular neurons, by increasing their expression of AVP. The possibility that neurons might have the capacity to up-regulate protein gene expression in response to a stressor is not a new concept. Sawchenko et al. (1987) showed that CRF and AVP are present in the same neurosecretory granules of fibers found within the external zone of the median eminence, and AVP immunoreactivity increases within the parvocellular division of the PVN in response to adrenalectomy (Kiss et al., 1984). In the case of neurohypophysectomy, however, few if any parvocellular fibers are injured because they terminate within the median eminence and not the neurohypophysis.

Our studies revealed no enhancement or compensatory expression of AVP immunoreactivity within parvocellular neurons or any other hypothalamic or accessory cell group. It still remains possible that the cells of the parvocellular division are indeed synthesizing AVP but transporting at such a rate to the median eminence for release that it is undetectable. It would be interesting to follow neurohypophysectomy with an injection of ibotenic acid into the area of the PVN. Ibotenate has been shown to preferentially kill parvocellular neurons at low doses (Herman et al., 1986). Such a loss would enable one to separate the effects of magnocellular neurons and parvocellular neurons in response to axotomy.

In addition to observing AVP expressing cells which survive following axotomy, immunohistochemical procedures were used to simultaneously label cells for oxytocin as well as vasopressin following insult. While the one-neuron-one hormone concept for AVP and OXY expression is highly favored by most researchers within this field, the remarkable plastic nature of oxytocinergic cells in response to a whole host of stimuli made exploring the possibility that AVP and OXT are co-expressed within the same neuron interesting. We found a very small population of doubly labeled cells within the medial, ventral portion of the PVN and lateral SON but no significant clustering or

number of such cell populations. Several cells within the retrochiasmatic nucleus did, however, label for both AVP and OXY. More animals need to be analysed ($n = 2$ Fig.14) before any conclusions may be drawn but this finding does seem to support findings by Sokol et al. (1976) and Baldino et al. (1988) that some magnocellular neurons have the capacity to synthesize AVP and OXY under certain conditions.

We also observed an extrahypothalamic population of cells lining the wall of the caudal portion of the third ventricle. These cells were not magnocellular neurons but astrocytes which colocalized for both GFAP and AVP simultaneously (Fig. 16). Again the number of immunopositive cells was small and would certainly not account for any marked amount of AVP release involved in the partial recovery of the vasopressinergic system but these data, in addition to several other reports, suggest the capacity of several different cell types of the CNS have the capacity to synthesize peptides *de novo* in response to various stimuli e.g. neurohypophysectomy and salt loading.

THE MODEL AND ITS USEFULNESS

Neuronal regrowth in the CNS is surprisingly pervasive and only recently has this begun to be appreciated. Establishing an *in vivo* model of central neuronal regeneration to identify functional markers of synaptic plasticity, growth factors, gangliosides, and/or trophic factors directly involved in axonal reorganization may ultimately lead to the development of therapeutic agents. The hypothalamo-neurohypophysial (HNH) system is a unique part of the CNS which has been noted for its remarkable plasticity following axotomy. The regeneration is not neuro-neuronal but neurohemal. These specialized contacts are, however, morphologically and functionally similar to true synapses and have been shown to be in every way comparable to the specializations found in neuro-neuronal synapses (Raisman, 1973). Thus this extension of the CNS provides an

excellent opportunity in which to ask and test a variety of questions concerning axonal regeneration, neuronal response to various stimuli and ultimately the ability of these CNS neurons to re-establish their functional abilities.

Unlike other areas of the CNS known to be plastic, e.g. the hippocampus, the HNH system allows one to easily assess the restoration of function within the system, namely water balance and AVP release. It presents enormous potential toward the understanding of nervous regeneration within the CNS and may indeed be a very credible *in vivo* model - notwithstanding the presence of two caveats: 1. The extent of cellular damage within the primary nuclei (SON and PVN) of the neurosecretory system is highly variable following axotomy. Of note, this study, in addition to previous studies, confirms that removal of the hypophysis is difficult to control with regard to the extent of axonal trauma. This, in part, is the cause of a varied range of physiological findings reported throughout the literature. 2. Plasticity in this system does not involve neuro-neuronal synapses but neurohemal contacts.

When using the model to study only those neurons of the HNH system which project to the neurohypophysis, removal of the entire pituitary gland (anterior and posterior lobe) yields multiple interpretations. The effect of the hypothalamo-pituitary-adrenal (HPA) axis on the SON and particularly the PVN has been well documented. Loss of the anterior lobe, thus the many hormones associated with it, affects the hypothalamic neurons in various ways. A better controlled model system in which to study the axonal regeneration of the HNH system and avoiding any complicating factors due to systemic changes in the secretion of such hormones as growth hormone, prolactin, leutinizing hormone, thyrotrophin stimulating hormone, etc. is neurohypophysectomy. Cell loss is not as dramatic as in the case of hypophysectomy (Figs.7 and 8) and anterior hormone secretion remains functioning thus eliciting no additional effects on severed

hypothalamic neurons. Unfortunately, studies which involve the removal of the neurohypophysis exclusively are not trivial. Neurohypophysectomy using a parapharyngeal approach is a very delicate and in our hands resulted in a high mortality rate (~80%). Recently, however, a new surgical procedure has been introduced in which degeneration of magnocellular nerve terminals in the neurohypophysis was induced by compressing the pituitary stalk. While this model has several advantages for physiological studies of pituitary function in rats, the persistence of the target organ results in a regrowth of axons toward the original site of contact. No reorganization of a "mini neural lobe" within the proximal part of the infundibulum and median eminence occurs. While it may be argued that axonal growth into the target organ may be responding to the same cues as axons re-investing the fenestrated capillaries of the external zone, this has yet to be proven. It may well be that the absence of a blood-brain barrier around the gland and median eminence does indeed carry with it the same peripheral cues or trophic factors required for axonal growth, elongation, and terminal specialization. Of note, there have been some studies which have suggested that the characteristic vessel hypertrophy and growth of a new mini neural lobe is inhibited by denervation of sympathetic tissue such as cervical ganglia.

As previously stated by Raisman (1973) and Dellman (1973), it is important to acknowledge that what we learn from this model system may or may not be representative of most other areas of the CNS. Neurons of the HNH system are unique in several ways. The presence of fenestrated capillaries, hence the absence of a blood-brain barrier, is unique as are the neurohemal associations made by the secretory neurons. Many laboratories are currently investigating the influence of peripheral factors on the reorganization of neurons within this system.

FUTURE PERSPECTIVES

We are far from identifying all the factors associated with the structural plasticity of each hypothalamic center but many researchers have suggested that a potentially fruitful avenue of investigation is to assess whether hypothalamic neurons and/or glial cells express proteins believed to be putative markers for histogenesis in developing neuronal systems. At the basis of such an approach is the belief that the processes underlying structural reorganization of the injured, adult CNS may be possible due to the up-regulation of embryonic gene programs for protein expression (Dellman, 1973, Tetzlaff et al., 1993, Geddes et al., 1990). Villar et al. (1990) and others have begun to use immunohistochemistry to identify putative peptides thought to be involved in either axonal growth or synaptic plasticity. Using the HNH system under control and hypophysectomy conditions, numerous factors have been identified within the magnocellular neurons of the HNH system, but few have been shown to be essential to the process of axonal regeneration. Recently in the laboratory we have begun studies whose ultimate goal is to block to expression of suspected plasticity and growth factors involved in the remodeling and axonal regeneration of the vasopressinergic system. The plan is to use adenovirus laden with antisense oligonucleotides for various plasticity factors and inject the virus directly into the neurohypophysis. Once the virus has been taken up by fibers of the neurosecretory system, the neurohypophysis of the animal will be removed and the animal will be monitored for at least 28 days. Water and urine values will be monitored as well as the animals' ability to release AVP in response to hemorrhagic-like stress. Thus far we have been able to inject adenovirus particles containing the reporter gene for b-galactosidase (β -gal) into the neurohypophysis of several rats. We have been able to detect the presence of β -gal expression within SON and PVN cells as early as two days and as late as 28 days following injection. It is also

possible that neurohypophysectomy may result in an even more robust signal in these animals as the magnocellular neurons have been described as increasing their cellular activity in response to axotomy. Potential gene candidates include AVP itself, T α -1-tubulin, epidermal growth factor (EGF), etc. Does AVP play an essential role by influencing its own release? One would assume yes, because work of Herman et al. (1986) showed that exogenously added AVP prevented even the partial recovery of the neurosecretory system. T α -1-tubulin mRNA has been shown to increase in response to neuronal axotomy and is down regulated at the approximate time of target contact (Miller et al., 1989). Whether its synthesis is essential to axonal regeneration may be directly addressed as this work is continued in the laboratory.

In summary, this thesis has utilized descriptive and qualitative visual observation to characterize some key components involved in the reorganization of the HNH system following hypophysectomy or neurohypophysectomy, namely; the time frame involved in the partial restoration of the HNH system, the confirmation that virtually all cells of the SON and PVN project to the neurohypophysis, and another piece of evidence which suggests that cells other than vasopressinergic neurons may in fact be capable of expressing AVP in response to axotomy. This work has provided a substantial foundation for the functional identification of other factors involved in neuronal reorganization of the vasopressinergic system.

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